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SECTION MEETINGS

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## Comparative Liberation of Bound Phosphatides from Red Cells of Man, Ox, and Camel.* (24415)

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It has recently been reported that lecithin is lacking from stromata of red blood cells of the ox, sheep, and goat(1). On the other hand, cells of 8 species of non-ruminants all contained substantial amounts of lecithin. The findings could be correlated with susceptibility to direct hemolysin of cobra venom. The cells of most animals are lysed by venom but those of true ruminants (ox, sheep, goat) are completely resistant. The venom toxin appeared, therefore, to be a lecithinase, and susceptibility to its action dependent upon presence of lecithin in the cell membrane. It was also noted that the erythrocytes of the *Pecora* are distinguished from those of other groups of mammals by additional peculiarities including their remarkably small size(2). From a morphological point of view it became of particular interest to examine the phosphatides of red cells of the camel, since they are unique among mammalian types in being oval, rather than circular, discs. When washed red cells of a dromedary and of a guanaco were tested for susceptibility to direct hemolysin of cobra venom they were quite resistant to lysis. The finding suggested that camel red cells might lack lecithin. However, when lipid extracts of both dromedary and guanaco cells were made and subjected to paper chromatography, large spots corresponding to lecithin were found(1), as well as other spots indicating presence of appreciable amounts of cephalins and of sphingomyelin. Enough material has not yet been obtained for a properly detailed analysis of the lipids of camel cells. Some additional evidence was secured nonetheless by allowing venom to act on lipid extracts according to the method of Hanahan (3). When chromatography was subsequently carried out, it was found that most of the lecithin and the cephalins had disappeared, to be replaced by spots of slower mobility corresponding to lysophosphatides. These preliminary data indicated that lecithin is probably present in the camel red cell and that resistance to venom in this case may be due to a binding of lecithin in such a way that it is "unavailable" to venom lecithinase(4). The experiments now reported represent an attempt to examine comparative stability of li-

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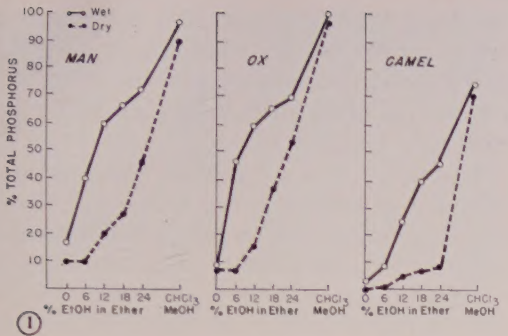
pid complexes of red blood cells. It will be shown, with a system of extraction by ether-alcohol mixtures, that the stromal phosphatides are extracted much less readily from camel cells than from either human or bovine cells. The method of extraction employed derives from that of Macheboeuf as applied to plasma lipoproteins(5), and was adopted on the suggestion that similar studies might be made of various tissues(6).

*Methods.* The red cells of citrated or oxalated fresh whole blood were washed 3 times in 0.9% NaCl and the buffy coat removed. Lysis was carried out with 10 to 20 volumes of water and the ghosts collected by centrifugation. These were now washed several times in 20 volumes of water, until the supernatant was phosphorus-free. The washed ghosts were then lyophilized and stored at –20°C until used. Extractions with ether-ethanol were carried out at 4°C on 100 mg lyophilized ghosts containing a total of 200 to 300 $\mu$g P. Separate studies were made of the influence of water by adding 0.3 to 0.4 ml $H_2O$ to 100 mg lyophilized ghosts. In both cases the material was extracted by mixing in 50 ml centrifuge tube with 25 ml solvent. This was centrifuged and the supernatant collected after filtering. An additional extraction with 25 ml of the same solvent was carried out in the same way and the supernatants were pooled. The sedimented ghosts were then extracted with the next solvent. The first solvent was anhydrous diethyl ether, and this was followed by a series of increasing amounts of absolute ethanol in ether, *viz.*, 6%, 12%, 18%, 24%. Finally, a last extraction was performed with $CH\ Cl_3$ : Me OH (2:1) at room temperature. Studies were also made using a series of ether-methanol mixtures at room temperature. Phosphorus was determined by the method of Beveridge and Johnson(7) after appropriate concentration of the extracts. The effectiveness of the lyophilization process was examined several times by further drying over $P_2O_5$ and the additional loss of water was never more than 3% of weight. Chromatography on silicated papers of concentrates of many of the extracts was carried out as previously described(1). It should be emphasized that extractions were carried out as quickly as possible, *i.e.*, time elapsing between first exposure of ghosts to a solvent and their transfer to the next solvent was about 30 minutes. This procedure was adopted on the view that the shorter the time the less the degree of denaturation. Some clumping of ghosts occurred, particularly in the solvents containing little or no alcohol. In separate experiments with human ghosts macroscopic clumping was appreciably reduced by mixing and grinding in a mortar the dry ghosts with twice their weight of Celite, before extraction. This step did not result in increased removal of phosphatides by the ether-alcohol mixtures. Furthermore, the Celite remained with the final residue of ghosts and interfered with P determinations on those residues. It was concluded that use of inert dispersing agents was likely to aid little in increasing the solvents' accessibility to the cells and might well introduce new errors. Moreover, the simple method of direct extraction of the ghosts was validated by the reproducibility of the results and the magnitude of the differences that could be shown between camel cells and those of other species.

*Results.* Fig. 1 exhibits the principal data. It can be seen that for lyophilized human and bovine cells very little phosphatide is removed until the percentage of ethanol reaches 12%. Thereafter appears a steady rise in the curve, so that with completion of the last (24%) ethanol extraction some 45 to 55% of the total P has been removed from the cells. On the other hand, the corresponding point on the curve for camel cells gives a value of less than 10%.

When the cells were moistened with water before extraction, liberation of phosphatides was enhanced in all cases. Again, however, the curve in the case of the camel is less steep than for man and ox. Thus, for example, at the 12% ethanol level, 24% of the P has been extracted from camel cells, whereas 60% has been removed from human and bovine cells. When lyophilized cells were extracted at room temperature with ether-methanol mixtures, similar results were obtained (Fig. 2). Rate of liberation of phosphatides from camel cells once more lags behind those of the other animals.



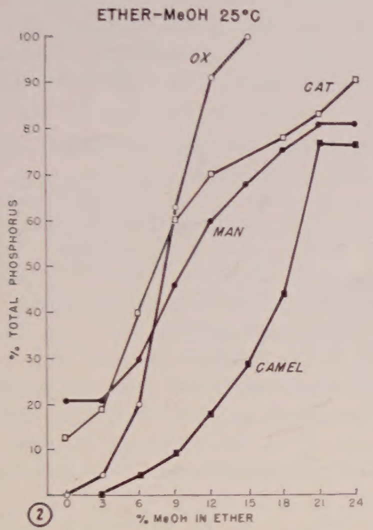


FIG. 1. Comparative rates of liberation of phosphatides from red cell ghosts. Ordinate: % of total P extracted. Abscissae: Progressive series of extractions with solvents as noted. Ether-ethanol at 4°C; $CH\,Cl_3$: methanol (2:1) at 25°C. Dotted lines: Lyophilized ghosts. Solid lines: Wet ghosts.

FIG. 2. Liberation of phosphatides from lyophilized red-cell stromata by ether-methanol mixtures at 25°C.

In view of Chargaff's studies of complexes of acidic phospholipides, *e.g.*, phosphatidyl serine(8) it became of interest to see whether the neutral choline-containing phosphatides were liberated from red cells more rapidly than the cephalins. The paper chromatograms made of the various extracts of human and bovine cells were all that was attempted in this respect and no differences in rates of release of the individual phosphatide components were observed. No attempt was made, however, to distinguish phosphatidyl serine from phosphatidyl ethanolamine. In separate experiments with human stromata 0.1 M acetic acid was incorporated in one set of solvents and 0.1 M pyridine in another; the curves of removal of P were much the same as those of Fig. 1, and the paper chromatographs of the extracts showed no preferential removal of choline-containing as opposed to amino-containing lipides.

As for the effect of water in accelerating release of lipid into organic solvents (Fig. 1) it is worth noting that this was accompanied by appearance of "proteolipid" which was not encountered in extracts of dry ghosts. In separate experiments direct extractions of wet and of dry stromata by chloroform-methanol (2:1) were compared. When extracts of the wet material were evaporated *in vacuo* to dryness and the residue then taken up in chloroform-methanol an appreciable amount of insoluble brownish-red material was encountered. This corresponds to the protein of Folch's "proteolipids," seen with great regularity in extractions of wet tissues(9). Extracts of dry ghosts were free of such protein even though the removal of total phosphorus of the cell was virtually complete, and it would appear that at least for red cells "proteolipid" may represent under these experimental conditions an artefact of denaturation rather than a biochemical entity.

*Discussion.* The findings could be explained either in terms of a firmer binding of the lipids of the camel ghost, or as a manifestation of some fundamental difference in orientation at the surface. Thus, recent studies of the human red cell ghost lead to the view that the lipid is disposed at or near the outside of the membrane(10). Perhaps in the camel this is not true, and, if further, the structural arrangement were such as to restrict the penetration of organic solvents, the data could thus be explained essentially in mechanical terms. But the lyophilized material appears microscopically to be largely amorphous, and the shape of the native cell is not seen after addition of saline. Also, the curious effect of water in accelerating liberation of lipid (Fig. 1) applies to the camel as well as to human and bovine cells. This could conceivably involve opening of pores even in the denatured membrane, but such a mechanical operation is probably not the whole story. It has been pointed out, for instance, that "proteolipid" appears in extracts of wet ghosts, and this would be difficult to account for on a completely mechanical view. It seems most probable that the experimental extraction procedures employed do give an indication of relative stability of certain undefined lipid complexes in the red cell membrane. The data may therefore be regarded as evidence for the view that insusceptibility of camel cells to cobra hemolysin is due to a peculiar binding of lecithin which makes it inaccessible to venom enzymes. The idea has been advanced many times in connection with the old observation that the red cells of some species, notably the ruminants, are resistant to venom(4), but never examined experimentally. In any case, either the binding or the spatial orientation of red cell phosphatides, or both, would appear to be different in the camel. At the same time it is clear that detailed analysis of these lipids is desirable; the possibility exists, however unlikely, that the findings may be further related to a novel sort of phosphatide.

It is impossible to say whether phosphatide composition or structure may be related to the morphological features of size and shape. But it can be noted (a) that appreciable amounts of lecithin cannot be found in the red cells of the 3 species of true ruminants thus far examined, while the cells of this group as a whole are relatively small in size, and (b) that a peculiarity of lipid constitution appears to exist in the camel cell, which has an oval shape.

The method of extraction may perhaps, with suitable modifications, be applied to pathological tissues, *e.g.*, in human diseases involving the erythrocyte it is possible that lesions of the red cell membrane could exist that might be manifested in terms of the ease or difficulty with which bound lipids are liberated.

*Summary.* The red cells of the camel are like those of true ruminants in being resistant to lysis by cobra venom but dissimilar in appearing to contain lecithin. The idea that the resistance of camel cells to venom is due to the binding of lecithin so that it is inaccessible to venom enzymes has been tested in terms of comparative extractability into ether-ethanol mixtures of phosphatides from cells of man, ox, and camel. The lipids were liberated much less readily in the case of the camel, indicating that the lipid complexes are different and more stable.

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## Effect of Reserpine* on Mammary Gland Involution, and on Other Organs in the Rat. (24416)

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A number of workers have reported recently on lactogenic effects of reserpine in several species, *i.e.* rabbits(1,2), rats(3,4) and man(5,6). However, in this laboratory, Tindal(7) has investigated mammogenic or

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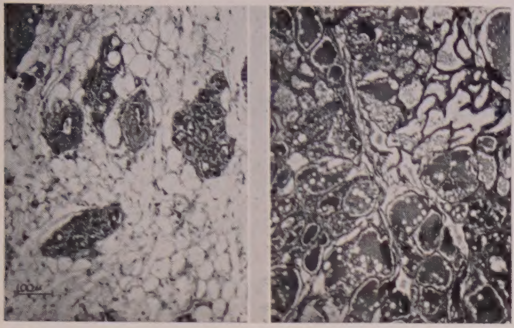

FIG. 1. Sections from left abdominal mammary glands of lactating rats treated with saline (left), and reserpine (right), 100 μg daily, for 9 days after removal of the pups on 4th day of lactation. Involution is retarded after treatment with reserpine.

lactogenic potency of this compound in rats, rabbits, goats and pigeons, without obtaining any positive effects. In view of the somewhat conflicting evidence with regard to effects of this substance on mammary gland, it was of interest to study the effect of reserpine on mammary gland involution in the lactating rat after weaning, as investigated by Benson and Folley(8). These authors have demonstrated that oxytocin, like prolactin, will inhibit mammary gland involution in rats after weaning, and it seemed possible, in view of the alleged lactogenic action of reserpine, that the administration of this substance would inhibit mammary regression in a similar manner.

*Materials and methods.* 24 Hooded Norway rats were used, 2-3 months old, undergoing first lactation. Stock colony diet(9) with addition of powdered milk, was fed *ad lib,* and food consumption was recorded daily. After parturition all litters were reduced to 9 pups on first day of lactation, and to 8 on second day. Litters were weighed daily to check lactational performance of the mother, and the mothers were also weighed daily. Pups were removed on 4th day of lactation, when treatment began. Reserpine was administered, as supplied, in a stabilised aqueous vehicle. The animals were divided into 3 groups of 8, one group receiving 50 μg reserpine daily; the second group 100 μg reserpine daily, and the third group served as a control and received 0.5 ml saline daily. The substance was in all cases administered as a single daily injection, subcutaneously, and treatment was carried out for 9 days, the animal being killed on the 13th day after parturition. Histological study of mammary tissue was then carried out exactly as described by Benson and Folley(8), the estimation of percentage parenchyma again being used as a quantitative method of assessing alveolar maintenance. In addition various other organs were removed, weighed, and preserved for further study.

*Results.* The mammary glands of both reserpine treated groups exhibited a very high degree of alveolar maintenance, which was particularly marked in the group receiving highest dose of reserpine. This is in contrast to the advanced state of regression observed in mammary glands of the saline treated group (Fig. 1). In Table I the values for percentage parenchyma of all 3 groups are given; the figure of 67.4 for the higher dosage group is remarkably high, being comparable to the best results obtained in this type of experiment, after treatment with a combination of prolactin and growth hormone (STH)(8).

*Vaginae, uteri and ovaries.* Uteri of untreated animals were significantly heavier than those from the reserpine treated groups, but there was no significant difference between weights of ovaries (Table I). Histological examination of uteri and ovaries from all 3 groups has also been carried out. No apparent differences could be detected in the ovaries, but the observations made on uteri indicate that ovaries differed functionally, and suggest an explanation for uterine weight differences. Thus, a number of uteri from untreated group exhibited a prooestrous or oestrous condition(10), a finding which was confirmed when the vaginae from the same animals were examined. In contrast, vaginae of all reserpine treated animals showed marked vaginal mucification, which compares favorably with results reported by Benson and Folley(8) after oxytocin administration, and appears to confirm the assumption that prolactin (LTH) has been released as a result of reserpine treatment. Evidence for luteotrophic activity following reserpine administration has previously been reported by Barraclough(11).

TABLE I. Effect of Reserpine Treatment on Mammary Gland Regression, Body Weight, Organ Weights and Food Consumption, in the Rat after Weaning at the Fourth Day of Lactation; 8 Rats per Group.

| Treatment | % parenchyma at 13th day | % decrease in body wt over treatment period | Mean daily food consumption (g) over treatment period | Mean wt (mg)/100 g body wt | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | Thymus | Adrenals | Uterus | Ovaries | Thyroids |
| Saline, .5 ml daily | 23.2 ± 3.4 | 1.0 ± .9 | 16.7 ± .6 | 131.8 ± 6.9 | 29.2 ± 1.0 | 191.5 ± 7.1 | 35.8 ± 1.9 | 12.7 ± .9 |
| Reserpine, 50 μg daily | 54.0 ± 4.6 | 3.0 ± 1.3 | 16.6 ± .6 | 100.1 ± 12.5 | 34.2 ± 1.5 | 115.2 ± 10.3 | 32.7 ± 1.2 | 12.3 ± 1.0 |
| ” ,100 ” ” | 67.4 ± 3.9 | 22.0 ± 2.9 | 8.9 ± .9 | 56.9 ± 5.3 | 41.8 ± 2.3 | 137.9 ± 6.7 | 33.4 ± 1.4 | 13.3 ± .6 |

*Other organs.* A study of organ weights of the 3 groups (Table I) reveals that reserpine has caused a highly significant increase in adrenal weight over the 9 day treatment period, as compared to control values, with a corresponding highly significant decrease in thymus weight. This is again particularly marked in the group receiving higher dose of reserpine. There is no significant difference between weights of thyroids of treated and untreated groups.

*Body weight.* The smaller dose of reserpine had little effect on food intake and body weight over the treatment period when compared to the control group. The higher dose, however, greatly affected appetite, the food intake being approximately halved, and a much larger percentage body weight decrease was apparent in this group over the treatment period (Table I).

*Discussion.* The results of this experiment appear to be in harmony with the conclusion of several authors that reserpine exerts a lactogenic effect under certain experimental conditions(1-6). Its effect in this experiment parallels that of synthetic oxytocin or a combination of prolactin and STH administered under similar circumstances(8). How this effect is obtained is not clear however. It is possible, as suggested by several authors (1,3,4) that reserpine stimulates release and/or secretion of prolactin by the anterior pituitary. On the other hand, since reserpine is considered to exert its general depressant effects by way of the central nervous system (12) it might be postulated that its lactogenic effect is due to its action in suppressing an inhibitory centre in the hypothalamus controlling release of prolactin. Such a centre has been postulated for the control of FSH release(13). Finally, of course, there is the possibility that the effect of reserpine is due to a direct action of the substance on the mammary gland, though this seems unlikely in view of the supplementary observations made on vaginae and uteri. Further experiments are in hand, however, to investigate these possibilities.

The pronounced effect of the compound on thymus and adrenal weights is of interest, and it would appear that in this experiment reserpine has markedly stimulated ACTH release. This is in agreement with the findings of Gaunt *et al.*(12) in the rat, and Harwood and Mason(14) in the monkey, though it has also been suggested that reserpine will block the stress induced release of ACTH in the rat, provided that steps are taken to block its initial stimulatory action (Wells, Briggs & Munson,15). These authors observed no change in adrenal weight as a result of reserpine administration, a finding also reported by Fluhmann(16).

The apparent effect of reserpine in causing a discharge of ACTH in this experiment is unlikely to have had any significant effect on mammary gland regression however, since it has recently been demonstrated that ACTH administration has only a slight effect on this process in normal lactating rats after weaning(17).

The absence of any effect on thyroid weight is probably of little significance in view of the comparatively short treatment period, though Fluhmann(16) has reported a marked decrease in thyroid weight following reserpine administration to young rats over a period of 44-49 days.

*Summary.* 1. The effect of reserpine on mammary gland regression after weaning in the rat has been studied. Two groups of 8 lactating rats received 50 μg and 100 μg of reserpine respectively for 9 days after removal of pups on 4th day of lactation. A third group of 8 animals which received saline injections served as a control. Histological examination of the mammary glands at the end of this period revealed that reserpine administration caused a marked inhibition of mammary involution, this being particularly pronounced at the higher dose level. 2. Reserpine also caused a pronounced decrease in food intake and a considerable loss in body weight at the higher dose level. 3. Both levels of reserpine administration resulted in a significant increase in adrenal weight and a concomitant decrease in thymus weight. Uterine weight was also reduced but ovarian and thyroid weight was unaffected. 4. Histological examination of vaginae and uteri revealed that whereas all reserpine treated animals showed evidence of LTH release (vaginal mucification), several of the untreated animals were cycling normally again, their uteri and vaginae exhibiting prooestrous or oestrous conditions. 5. These results are discussed in relation to the possible mechanisms by which reserpine may exert its effects.

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# Purification and Isolation of Rheumatoid Factor. (24417)

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Serum of patients with rheumatoid arthritis has been shown to agglutinate bacteria (1) and particles or cells sensitized with human or rabbit gamma globulin. Sensitized sheep erythrocytes(2) have been commonly used to demonstrate activity of rheumatoid agglutination activating factor. Partial purification has been achieved by column chromatographic separations of serum proteins of rheumatoid serum on anionic and cationic cellulose ion exchangers(3). Also purification has been achieved by dissociation of complexes precipitated from rheumatoid serum by heated human Cohn Fraction II preparations followed by differential ultracentrifugation through a sucrose density gradient(4). Svartz *et al.*(5) used cold precipitable globulins of rheumatoid serum as starting material for fractionation on carboxymethyl cellulose to yield an apparently homogeneous protein with a sedimentation coefficient of 18.7. Concentration of one of the preparations(5) was too low to demonstrate homogeneity in the ultracentrifuge unequivocally, and no concentration dependence data were given for the sedimentation coefficient. The other preparation (4) was obtained after dissociation at pH 3.0. At this reaction the material eventually loses all activity(3). As a result, even brief exposures to pH 3.0 buffers might be expected to produce some denaturation of the rheumatoid factor. It, therefore, seemed useful to present some data on an active material prepared by a different method. Furthermore, we are able to present some precise quantitative data(6,7) on the relative specific activities of the material at successive stages of purification.

*Methods and materials.* Relative specific activities were determined by a method we developed, which measures inhibition of hemolytic activity of sensitized Newcastle Disease Virus particles by the rheumatoid factor(6,7). By using this method relative specific activities of the material at successive stages of purification with a precision of ± 5% for duplicate samples can be determined. The method of fractionation proceeded as follows: 500 ml of serum derived from 550 ml of rheumatoid plasma by thrombin treatment was dialyzed against 0.01 M phosphate pH 5.8. The resulting euglobulin was redissolved in phosphate buffered isotonic saline and dialyzed with 2 g of diethylamino-ethyl (DEAE) cellulose against 0.01 M phosphate pH 7.9. 93% of the activity was adsorbed. The DEAE and adsorbed euglobulin were transferred to the top of 18 g DEAE column for elution by stepwise increased salt and decreased pH method of Lospalluto and Ziff (3). An active peak emerged during elution with 0.15 M phosphate pH 5.0. An aliquot of this material was adjusted to 0.1 M phosphate pH 7.4 by dialysis after freeze drying. The remainder was dialyzed against 0.1 M phosphate pH 4.4. After concentration by freeze drying to 30 ml, 20 ml of this solution was chromatographed on 10 g of carboxymethylcellulose (CMC) equilibrated with pH 4.4 phosphate. A sharp peak containing the activity emerged at pH 4.4. This material was freeze dried and dialyzed against 0.1 M phosphate pH 7.4.

*Results.* Fig. 1 shows sedimentation patterns of DEAE activity peak and CMC activity peak. The DEAE material shows 4 components with sedimentation coefficients of approximately 7, 9, 19, and 30. The CMC eluate shows at least 90% of $S^{\circ}_{20,w}$ 18.8 com-

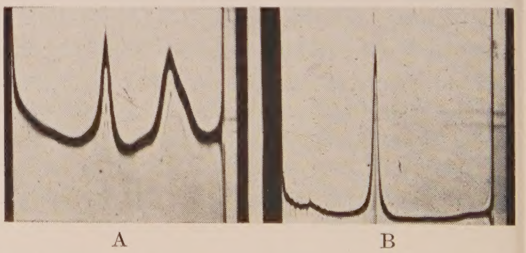

FIG. 1. Sedimentation diagrams of rheumatoid factor preparations: (A) Activity peak eluted from DEAE. (B) 1A after elution from CMC at pH 4.4. Both pictures taken after 24 min. at 59,780 r.p.m.

TABLE I. Sensitized NDV Hemolysis Inhibition Activity.

| Fraction | Units/ml | Nitrogen (mg/ml) | Specific activity (units/mg N) | Specific activity × serum |
|---|---|---|---|---|
| Serum | 37 | 11.2 | 3.3 | 1 |
| Euglobulin | 118 | .82 | 143 | 43 |
| DEAE, pH 7.4 | 628 | .98 | 641 | 194 |
| " , pH 4.4 | 656 | 1.08 | 607 | 181 |
| CMC | 1751 | .98 | 1786 | 543 |

ponent and a small component with $S^{\circ}_{20,w}$ greater than 30, possibly an aggregate of the 18.8 component. A trace of slow material is also seen. The peak is sharper and more homogeneous in appearance than the material prepared by Edelman *et al.*(4).

Relative specific activities at successive stages of purification are shown in Table I. Fourteen per cent of the nitrogen and 39% of the activity were recovered from the CMC column. The material shown in Fig. 1B had a sensitized sheep cell agglutination titre of 1:5120.

Fig. 2 shows the data on concentration dependence of sedimentation coefficient, and the extrapolated value of 18.8 S at zero concentration.

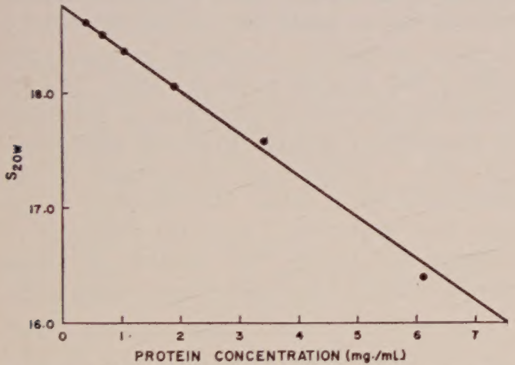

FIG. 2. Concentration dependence of sedimentation coefficient of major peak from Fig. 1B.

*Summary.* A method for purification of the rheumatoid factor has been presented. A euglobulin fraction of serum was chromatographed on DEAE cellulose. The resulting active fraction was further chromatographed on carboxymethyl cellulose to yield a preparation which contained more than 90% of a component with a sedimentation coefficient of 18.8. Relative specific activities at successive stages of purification were determined. Concentration dependence data for the sedimentation coefficient were obtained.

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# Production and Absorption of Organic Acids by the Perfused Goat Rumen.[*] (24418)

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It is well established that large quantities of volatile fatty acids (VFA) are produced within the rumen by microbial action on cellulose, starch and protein. One of the first requirements for an understanding of the biochemistry of ruminant metabolism is a knowledge of quantities and proportions of the VFA produced and absorbed and the form in which these rumen substances enter the blood of the host animal. Numerous technics have been devised to gain a measure of these values; however, these methods have measured only one part of this system, *i.e.*, either production or absorption(1). That variable results have been obtained is not unexpected in view of the dynamic nature of this system where production and absorption occur continuously and simultaneously. Rumen perfusion was selected as a technic for measuring simultaneously both total production and absorption of VFA.

*Methods.* The rumina of 2 mature goats weighing approximately 60 lb were perfused with heparinized blood by use of pump-oxygenator apparatus similar to one used previously in this laboratory(2). These animals had been fed a diet of mixed hay and concentrate *ad lib.* for 6 weeks prior to the perfusion studies. Labeled substrates were added to rumen contents immediately prior to perfusion, 50 $\mu$c of n-butyrate-1-$C^{14}$Na being used for perfusion 40 and 50 $\mu$c of propionate-1-$C^{14}$Na in perfusion 42. The 2 perfusions, 40 and 42, were conducted for 80 and 115 minutes respectively. Organic acids in blood and rumen fluid were measured by modification of the chromatographic technic of Wiseman and Irvin(3). Blood glucose was determined by the method of Somogyi(4). Analysis for total acetone bodies in blood was performed by procedure of Weichselbaum and Somogyi (5). Acetoacetic acid was isolated by method of Cavallini and Frontali(6). Measurements of radioactivity were made with a windowless flow tube used in the Geiger region. Osazones were prepared for estimating activity in blood glucose. The carboxyl carbon of $\beta$-hydroxybutyrate was removed by oxidation and counted as barium carbonate.

*Results.* Rates of blood flow through the isolated rumina were 65 ml/minute in perfusion 40 and 150 ml/minute in perfusion 42. These values are equivalent to 25 to 50% of the estimated *in vivo* rate(7).

Quantitative results for perfusion 40 are presented in Table I. The total increase in organic acids in the isolated system amounted to 2392 mg of which 31% is represented by increase in blood. Similarity in molar per cent of the organic acids in rumen fluid at beginning and end of perfusion, is indicative of maintenance of a nearly normal physiological state. Comparison of increases of individual acids in blood with increases in rumen fluid shows that, with the exception of formate and lactate, the increases in blood were a reflection of production of these acids.

The perfused rumen utilized glucose, blood concentration decreasing from 43.8 to 29.3 mg % during experiment. No change occurred in level of acetone bodies in the blood.

The quantitative results from perfusion 42 are presented in Table I. In an effort to remove vasaconstrictive substances blood was passed through the isolated liver of the goat for 10 minutes before attaching the blood supply to the vascular system of the rumen. This resulted in an increased concentration of certain blood metabolites.

The ruminal tissue utilized a total of 844 mg of glucose during perfusion, blood concentration decreasing from 265 mg to 171 mg/100 ml of blood. Blood levels of acetone

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TABLE I. Changes in Blood and Rumen Metabolites during Perfusion of Rumina of Goats.

| Blood and rumen metabolites | Organic acids in rumen liquor at beginning of perfusion | | Organic acids in rumen liquor at end of perfusion | | Increases in rumen organic acids during perfusion, mg | Total changes in blood metabolites during perfusion, mg |
|---|---|---|---|---|---|---|
| | mg/100 ml | Molar % of total | mg/100 ml | Molar % of total | | |
| Perfusion No. 40 (time, 80 min.)† | | | | | | |
| Glucose | | | | | | –145.0 |
| Acetone bodies* | | | | | | .0 |
| *Organic acids* | | | | | | |
| Valeric | 27.8 | 2.4 | 41.3 | 2.9 | 107.2 | 24.7 |
| Butyric | 139.8 | 14.2 | 173.5 | 14.4 | 267.9 | 106.5 |
| Propionic | 142.0 | 17.2 | 180.5 | 17.8 | 305.7 | 117.2 |
| Acetic | 397.0 | 59.9 | 477.8 | 58.3 | 641.2 | 362.6 |
| Formic | 4.8 | .9 | 6.1 | .9 | 10.3 | –32.9 |
| Lactic | 53.2 | 5.3 | 67.6 | 5.4 | 114.3 | 334.6 |
| Total organic acids | 764.5 | | 946.7 | | 1446.6 | 945.6 |
| Perfusion No. 42 (time, 115 min.)‡ | | | | | | |
| Glucose | | | | | | –843.8 |
| Acetone bodies* | | | | | | – 87.8 |
| *Organic acids* | | | | | | |
| Valeric | 10.1 | 1.1 | 11.2 | 1.0 | 18.4 | 18.0 |
| Butyric | 14.5 | 1.9 | 32.9 | 2.5 | 299.8 | 43.6 |
| Propionic | 152.2 | 23.3 | 229.5 | 21.0 | 1260.3 | 219.8 |
| Acetic | 391.4 | 73.8 | 669.2 | 75.7 | 4529.1 | 488.3 |
| Formic | .0 | | .0 | | .0 | –33.7 |
| Lactic | .0 | | .0 | | .0 | 154.9 |
| Total organic acids | 568.2 | | 942.9 | | 6107.6 | 924.7 |

* Acetone bodies calculated as $\beta$-hydroxybutyric acid. † Total volume: rumen fluid = 794 ml, blood = 1000 ml. ‡ Total volume: rumen fluid = 1630 ml, blood = 900 ml.

bodies decreased from 55.2 to 45.5 mg % during perfusion. As in the previous perfusion there was marked production of organic acids in the isolated system. However, in this case formate and lactate were not present in the rumen fluid.

Distribution of recovered $C^{14}$ label and specific activity (S.A.) of the various metabolites following perfusions 40 and 42 is shown in Table II. Distribution of label among the organic acids in rumen fluid at completion of perfusion 40, where labeled butyrate was added, (Table II) shows that there was recycling of the carboxyl carbon of butyric acid. Conversions of this nature have been reported previously(8,9). The greatest quantity of recovered label in blood was found in butyric acid. All blood acids measured, except formic acid, contained some of the $C^{14}$ label. Comparison of the S. A. of acids in rumen fluid with the same acid in blood shows that the specific activities of blood butyrate and lactate were higher than those of these same acids in the rumen. On the other hand, specific activities of acetate, propionate and valerate were greater in rumen fluid. Only a trace of label was found in $\beta$-hydroxybutyrate in blood while none was found in acetoacetate or blood glucose.

Distribution of radioactivity in the rumen at completion of perfusion 42 where labeled propionate was added, (Table II) shows that a minor amount of the propionate was converted into acetic and valeric acids. Within blood 97.3% of the recovered label was in propionic acid. It is interesting to note that formate in the blood perfusate was labeled even though there was utilization of formate by the ruminal tissue.

*Discussion.* Large quantities of organic acids are normally produced in the rumen. The results reported herein, where total production and absorption were measured, show that on a diet of hay and grain, relative rates of production of the VFA are acetate>propionate>butyrate>valerate. With the exception of formate and lactate, absorption of or-

TABLE II. Distribution of $C^{14}$ Label, and Specific Activity of Various Metabolites following Perfusion of Goat Rumina in Which n-Butyrate-l-$C^{14}$ Na or Propionate-l-$C^{14}$ Na Had Been Added to the Rumen Contents.

| Metabolites | Distribution of activity in rumen fluid, % | Distribution of activity in blood, % | Specific activity Rumen fluid (CPM/μm) | Specific activity Blood (CPM/μm) |
|---|---|---|---|---|
| Exp. 40—Labeled butyrate added* | | | | |
| Osazones | | | | 0 |
| Acetoacetic acid | | | | 0 |
| β-Hydroxybutyric acid | | trace | | 31 |
| $CO_2$ | | " | | |
| *Organic acids* | | | | |
| Valeric | 1.8 | 1.3 | 185 | 104 |
| Butyric | 61.9 | 72.4 | 742 | 1083 |
| Propionic | 10.6 | 8.4 | 100 | 97 |
| Acetic | 25.8 | 7.9 | 59 | 21 |
| Formic | .0 | .0 | 0 | 0 |
| Lactic | .4 | 9.7 | 14 | 25 |
| Exp. 42—Labeled propionate added† | | | | |
| Osazones | | .0 | | |
| *Organic acids* | | | | |
| Valeric | .7 | .7 | 5396 | 4048 |
| Butyric | .0 | .0 | 0 | 0 |
| Propionic | 95.5 | 97.3 | 26,689 | 35,870 |
| Acetic | 3.8 | 1.3 | 294 | 139 |
| Formic | | .8 | | 501 |
| Lactic | | .0 | | 0 |

* 81% of added $C^{14}$ recovered of which 6.3% was isolated in blood.

† 70% of added $C^{14}$ recovered of which 43.7% was isolated in blood.

ganic acids appears to be a reflection of their production.

Utilization of blood formate by ruminal tissue is consistent with the findings of Annison(10) who reported that in ruminants portal levels of formic acid were no greater than peripheral blood concentrations.

Distribution of label in the 2 perfusions shows that recycling of the organic acids in the rumen may occur. Conversion of butyrate to other acids was much greater than conversion of propionate. It is of interest to note that butyrate was converted to propionate (a pathway which is obscure at this time) while propionate was not converted to butyrate.

Comparison of the distribution of label and S. A. of organic acids in blood with distribution and S. A. of the same acids in rumen fluid indicates direct absorption of the rumen acids *per se*. The S. A. of butyrate in blood was greater than the S. A. of butyrate in the rumen at completion of experiment. If butyrate was being absorbed *per se* this would be the expected result. Initially all activity was in rumen butyrate and therefore butyrate absorbed in the beginning of experiment had a high S. A. During perfusion the S. A. of butyrate in the rumen decreased due to production of unlabeled acid and conversion of butyrate to other acids. Thus after a period of time the S. A. of butyrate in blood would be greater than the S. A. of this metabolite in the rumen. We have shown previously that butyrate does not pass from blood perfusate into the rumen(11). The data in perfusion 42 show that propionate was also absorbed as such from the rumen.

Direct absorption of acetate, propionate and valerate in perfusion 40 is supported by the fact that the S. A. of these acids in blood was lower than the S. A. of the same acid in the rumen fluid. In this perfusion the amount of label found in lactate present in blood perfusate suggests that it arises from an unknown mechanism in addition to direct absorption. This concept is also supported by comparison of S. A. of lactate in rumen fluid with that in blood.

In perfusion 42 neither rumen nor blood butyric acid were labeled and the blood lactate was not labeled. However, in perfusion 40 where both blood and rumen butyrate were highly labeled the rumen and blood lactate were labeled with the latter having the higher S. A. The greater S. A. of blood lactate indicates that a part of the blood lactate arose by a mechanism other than direct absorption. Black *et al.*(12) estimated that one-half to two-thirds of glucose catabolism in ruminants occurs *via* the pentose cycle. The pentose cycle involves oxidation and ultimate decarboxylation of glucose-6-phosphate to form a pentose. The further catabolism of the pentose yields a 2 and 3 carbon fragment. The 3 carbon fragment is usually lactic acid. It can be speculated that the apparent appearance of a small amount of label from butyrate in lactate could result from butyrate providing a 4 carbon intermediate which is known to be involved in the pentose cycle.

The appearance of label in blood formate in perfusion 42, even though there was a utilization of formate by the ruminal tissue, indicates that the labeled formate probably resulted from metabolism of the ruminal tissue. This fact shows that the rumen acids can contribute to ruminal tissue metabolism.

The large utilization of glucose in perfusion 42 and the decrease in blood levels of acetone bodies in this same perfusion most likely resulted from a mass action effect since the passage of blood through the isolated liver resulted in a marked increase in blood concentration of these metabolites.

The fact that there was no increase in blood levels of acetone bodies in either perfusion is contrary to the belief of most ruminologists who have interpreted the work of Pennington (13) as indicating that butyrate is absorbed in the form of acetone bodies. The latter noted quantitative conversion of butyrate to acetoacetate by ruminal epithelial tissue slices. In perfusion 40 in which labeled butyrate was added to the rumen contents only a trace of label was found in $\beta$-hydroxybutyrate in blood. Even though there was no net increase in blood levels of acetone bodies a comparison of the S. A. of blood butyrate with the S. A. of $\beta$-hydroxybutyrate makes it doubtful that a direct conversion was occurring. In several rumen perfusions in this laboratory a moderate increase in blood acetone bodies was noted(11). Davis *et al.* (abstract) however, did not obtain an increase in blood acetone bodies during their perfusion experiments.

Extrapolation to a 24 hour basis shows that the organic acid production of perfusions 40 and 42 could provide 31 and 53% respectively of the estimated daily net energy requirements of the animal.

*Summary.* 1) Any inferences drawn from data of perfusion experiments must obviously be tempered by artificial conditions under which the tissue is operating. While recognizing that there are limitations it seems that a picture of the overall metabolism of the rumen may be formulated. 2) Large quantities of organic acids are normally produced in the rumen. On a diet of hay and grain relative rates of production of the VFA are in the order acetate>propionate>butyrate>valerate. Lactate may also be produced. Calculations based on data from perfusion experiments indicates that the organic acids are capable of providing up to 53% of the estimated net energy requirements of the animal. 3) Interconversion of organic acids may occur in the rumen. Butyrate in the rumen was converted to appreciable amounts of acetate and propionate and to lesser amounts of valerate and lactate. The interconversion of propionate in the rumen was negligible. 4) Acids produced in the rumen are absorbed as such into the blood of the animal, and relative quantities of these acids absorbed are, in general, a reflection of their production in the rumen. Rumen acids do not serve as the immediate precursors of blood acetone bodies. However, these acids apparently can contribute in part to the metabolism of the ruminal tissue.

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# Metabolism of Volatile Fatty Acids by the Perfused Goat Liver.* (24419)

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Large quantities of volatile fatty acids (VFA) resulting from microbial activity in the fore-stomach of ruminants reach the liver directly by the portal vein. Experiments with rumen perfusions(1) demonstrated that the proportion of the VFA reaching the liver is very similar to the proportion in which these acids are produced in the rumen. Since the liver is capable of acting on all digestive products, it ultimately determines the nature and quantity of the metabolites presented to the other tissues of the body.

*Methods.* Livers of goats were perfused through the portal vein with heparinized blood for 1 hour periods by use of pump-oxygenator apparatus similar to one we previously used (2). Mixtures of VFA ($C_2$ to $C_5$) in proportions approximating normal absorption from the rumen were added to blood at beginning of each perfusion (Table I). In addition, in each perfusion a different carboxyl-$C^{14}$ labeled acid was added to blood as follows: formate, perfusion 43; acetate, perfusion 44; propionate, perfusion 45; butyrate, perfusion 46. Blood samples were taken as 0, 30 and 60 minutes after initiation of perfusion; initial and final liver samples were also obtained. Blood organic acids were determined by modification of the chromatographic technic of Wiseman and Irvin(3). Blood glucose was measured by method of Somogyi(4). Total acetone bodies were measured by technic of Weichselbaum and Somogyi(5). Liver glycogen was isolated according to procedure of Good *et al.*(6). Three fractions were extracted from liver tissue. A known weight of liver tissue was extracted with 1:1 ethanol-ether (v/v) 3 times in Waring blendor. The supernatant was combined and concentrated under reduced pressure. The ethanol-ether concentrate was then extracted 3 times with a large excess of warm hexane. Phospholipids were precipitated from the hexane extract by addition of acetone. The supernatant contained neutral fat. The remaining ethanol-ether material was extracted with acetone and the residual material from this extraction was taken up in moist ether. A phosphatide fraction was precipitated from the moist ether by addition of acetone and magnesium chloride. The precipitate was again taken up in moist ether and reprecipitated 3 times. The components of this phosphatide fraction were not isolated. A fraction known to contain Coenzyme A was prepared from another sample of liver tissue by the method of Lipmann *et al.* (7). This fraction had the same characteristics as the phosphatide fraction originally prepared, *i.e.*, a yellow, oily material. Also the fraction prepared by the method of Lipmann *et al.* had the same total radioactivity as the one previously prepared. All measurements of radioactivity were made with a windowless flow tube used in the Geiger region. Osazones were prepared for appraisal of activity in blood glucose. Blood acetone bodies were degraded into $CO_2$ and acetone. The 2,4 dinitrophenylhydrazones of the derived acetone were purified by procedure of Bassette (personal communication). The derived $CO_2$ was converted to barium carbonate for counting.

*Results.* In all experiments, flow of blood through the isolated liver attained the estimated *in vivo* rate(8) of 500 ml/minute. The quantitative results for all perfusions are presented in Table I. It will be noted that valerate, butyrate and propionate were removed almost completely from blood and metabolized by the liver. On the other hand, level of blood acetate increased moderately and there was a 2- to 3-fold increase in blood concentration of formate. There was an increase of 60 to 160% in blood acetone bodies. Blood glucose and lactic acid also increased markedly, undoubtedly due to glycolysis, since the increases were accompanied by a decrease in liver glycogen. Production of large quanti-

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TABLE I. Total Quantities of Metabolites in Perfusate and Total Amount of Liver Glycogen at Beginning and End of 4, One-Hour Goat Liver Perfusions.

| Metabolites* | Perfusion 43 | | Perfusion 44 | | Perfusion 45 | | Perfusion 46 | |
|---|---|---|---|---|---|---|---|---|
| | Initial | Final | Initial | Final | Initial | Final | Initial | Final |
| | (mg) | | | | | | | |
| *Blood* | | | | | | | | |
| Glucose | 775.6 | 2661.6 | 1065.0 | 5925.0 | 966.0 | 3780.0 | 630.0 | 3255.0 |
| Acetone bodies† | 166.3 | 275.1 | 278.4 | 445.4 | 256.1 | 534.5 | 228.2 | 579.1 |
| Valeric acid | 15.0 | .0 | 8.2 | .0 | 24.2 | .0 | 31.8 | .0 |
| Butyric " | 32.5 | .0 | 34.8 | .0 | 113.8 | .0 | 37.9 | .0 |
| Propionic " | 256.9 | .0 | 326.2 | 5.7 | 324.2 | 7.9 | 238.1 | .0 |
| Acetic " | 666.9 | 730.2 | 913.1 | 1056.5 | 880.7 | 975.8 | 650.4 | 715.2 |
| Formic " | 124.8 | 402.3 | 177.4 | 612.0 | 167.5 | 482.4 | 107.0 | 453.6 |
| Lactic " | 341.6 | 938.1 | 536.9 | 1508.9 | 755.5 | 1323.3 | 242.4 | 960.0 |
| *Liver* | | | | | | | | |
| Glycogen | 6870.0 | 5010.0 | 3605.0 | 1442.0 | 6542.0 | 4963.0 | 3910.0 | 2756.0 |

* Quantities of valerate, butyrate, propionate and acetate added to the liter of perfusate varied in each perfusion. Initial values reported in the table represent total added except for acetate where values represent quantity added plus amount already present in blood.

† Acetone bodies calculated as β-hydroxybutyrate.

ties of glucose is a characteristic of liver perfusions(9).

The data on distribution of recovered label from the carboxyl-labeled VFA at completion of perfusions are presented in Table II. The label from formate, propionate and butyrate recovered in liver glycogen, blood glucose and lactate accounted for approximately 64, 75, and 83% respectively of the total $C^{14}$ recovered. Only 12% of the recovered label from acetate was found in these compounds, most of the label remaining in the blood acetate.

TABLE II. Distribution of Recovered $C^{14}$ Label following 1-Hr Perfusions of Goat Livers in Which Carboxyl-Labeled Volatile Fatty Acids Were Added to Blood.

| Substance isolated | % distribution of label from added metabolite | | | |
|---|---|---|---|---|
| | Formate | Acetate | Propionate | Butyrate |
| *Blood* | | | | |
| Valeric acid | .0 | .0 | .0 | .0 |
| Butyric " | .0 | .0 | .0 | .0 |
| Propionic " | .0 | .0 | 1.6 | .6 |
| Acetic " | 2.1 | 75.2 | 3.6 | 10.3 |
| Formic " | 12.5 | 4.1 | 8.3 | 2.0 |
| Lactic " | 5.1 | 7.4 | 25.1 | 49.9 |
| Glucose | 1.3 | 2.4 | 3.1 | 1.1 |
| Acetone bodies: | | | | |
| Derived acetone | .0 | .0 | .0 | .0 |
| Derived carboxyl $CO_2$ | .6 | 1.5 | .6 | 1.1 |
| *Liver* | | | | |
| Glycogen | 57.8 | 2.4 | 46.5 | 31.5 |
| Neutral fat | .4 | 1.9 | .7 | .5 |
| Phospholipids | 5.6 | .4 | 1.1 | .2 |
| Nucleotide fraction | 15.1 | 6.0 | 10.0 | 4.0 |
| % added $C^{14}$ recovered | 61.9 | 71.6 | 50.1 | 90.0 |

Acetone derived from blood acetone bodies was not labeled and the $CO_2$ from the carboxyl group of β-hydroxybutyrate in blood was only slightly labeled. Since an increase of 10 to 20 mg% of total acetone bodies occurred in blood, it appears that acetone bodies must have been derived from lipids of liver which were only slightly labeled.

There was little lipogenesis in these 4 perfusions from the labeled substrates. The extent to which the VFA from the rumen may contribute to lipid metabolism has not been reported. The results here show that propionate and butyrate contribute more to carbohydrate metabolism than to lipid metabolism.

There was a fair degree of incorporation of the $C^{14}$ label into the phosphatide fraction. Incorporation was greatest from formate followed by propionate, acetate and butyrate. Isolation of the components of this fraction is necessary before the findings can be properly evaluated.

*Discussion.* The contribution of large quantities of butyrate to glycogen clarifies the paradoxical situation which existed until this time. Kleiber *et al.*(10) found that in the intact dairy cow there was appreciable labeling of milk lactose and casein from injected $C^{14}$ labeled butyrate. However, butyrate *per* *se* apparently contributed little to formation of milk fat. It has generally been believed that within ruminants, butyrate is metabolized as β-hydroxybutyrate(11). In studies at Maryland it was demonstrated with $C^{14}$ labeled β-hydroxybutyrate that this substance is converted into milk fat and protein but not into lactose by the perfused udder(12). With the demonstration here that butyrate is converted to glycogen by the liver the anomalies of these findings are removed.

It is also evident from these studies that butyrate was not metabolized as 2 acetyl groups. Since the known metabolic pathways show acetate and butyrate having a common intermediate, *i.e.,* Acetyl Coenzyme A, the activity distribution for both should have been similar if butyrate had been metabolized in this manner.

The contribution of propionate to carbohydrate metabolism has been demonstrated previously(13,14). The large quantity (75%) of the recovered label remaining in blood acetate indicates that acetate arising from the rumen is not altered appreciably by the liver but is made available as such to the extrahepatic tissues.

Production of formate by the liver explains the origin of formate normally found in ruminant blood. The results of rumen perfusions(1) showed that formate did not arise from the rumen; in fact blood formate was utilized by ruminal tissue. Annison(15) noted earlier that concentration of formate in the portal blood of sheep was no greater than the level in peripheral blood.

The low radioactivity in blood glucose can be explained by the fact that most of the increase in blood glucose occurred very early in the experiment before the labeled substrates were converted appreciably to carbohydrate. The greater total activity in blood lactate than in blood glucose indicates that the lactate was arising in a large part from glycogen in the tissue and not from blood glucose.

*Summary.* Formate, butyrate and propionate were removed from blood and metabolized by the perfused liver. Butyrate, propionate and formate contributed heavily to carbohydrate metabolism and formation of glycogen. Formate was produced by the liver, and even though size of formate pool increased considerably, there was substantial contribution of this pool to glycogen formation. Acetate levels in blood showed a slight increase during perfusion. The major portion of recovered label from added acetate remained in blood acetate indicating that most of the acetate arising from the rumen passes unaltered through the liver. The bulk of acetone bodies in ruminants apparently arises primarily from hepatic lipid metabolism and not from direct conversion of rumen VFA by the liver.

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# Effect of Aortic Constriction on Experimental Atherosclerosis in Rabbits.[†]

(24420)

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Clinical observation has shown that relief of arterial obstruction may be followed by progressive atherosclerosis of the distal arterial tree. Therefore it was decided to ascertain experimentally whether aortic constriction might lessen the production of distal atherosclerosis.

*Materials and methods.* Thirty adult female rabbits weighing 2-3 kg (5-7 lb) were utilized. Because of relative uninvolvement of the abdominal aorta in experimental atherosclerosis in the rabbit, a bilateral lumbar sympathectomy and periaortic adventitial stripping from renal arteries to aortic bifurcation was carried out in 20 animals. This has been shown by Murphy *et al.*(1) markedly to increase severity of abdominal aortic atherosclerosis in rabbits fed a high cholesterol diet. In 10 of the sympathectomized rabbits a silk ligature was then tied firmly over a No. 6 intestinal needle placed on the aorta at varying levels below the renal arteries. The needle was then withdrawn, leaving an aortic constriction approximately one-third the diameter of the aortic lumen. Ten rabbits served as non-operated controls. All animals were then placed on a pelleted diet as follows: Dehydrated alfalfa meal 96.5%; stabilized animal fat 1.5%; cholesterol U.S.P. .8%; toasted soya flour 1.2%. After 3-month interval, direct pressure tracings were made by introduction of a No. 20 needle into the femoral artery of unanesthetized rabbits and pressures were recorded with pressure transducer and direct writing oscillograph. The animals were sacrificed at 3 months and the entire thoraco-abdominal aorta and iliac arteries were removed. The vessel was opened along its longitudinal axis and fixed in 10% formalin for 24 hours. It was then stained in Scarlet Red solution better to delineate atherosclerotic areas.

*Results. Non-operated rabbits.* Average femoral artery pressure of these 10 rabbits was 122/94 mm Hg. The animals demonstrated almost solid coalescence of atherosclerotic plaques in the thoracic aorta but only occasional plaques in the lumbar aorta and iliac arteries (Fig. 1a).

*Sympathectomized rabbits.* Average femoral artery pressure in animals which underwent bilateral lumbar sympathectomy and periaortic adventitial stripping was 124/90 mm Hg. These animals all showed severe involvement of the lumbar aorta and iliac arteries as well as the thoracic aorta (Fig. 1b).

*Sympathectomized rabbits with aortic constriction.* Average femoral artery pressure in these animals was 84/72 mm Hg. These rabbits demonstrated severe atherosclerosis with coalescence of plaques in the thoracic and lumbar aorta to the level of constriction, but only occasional or rare plaques below that level (Fig. 1c, 1d).

Exposure of abdominal aorta of these animals under ether anesthesia revealed expansile pulsation of the vessel to the level of constriction, but no visible pulsation below that level. Direct pressure measurements above and below the constriction varied considerably because of anesthesia and trauma of the operative procedure, but in all cases demonstrated a marked diminution of pulse pressure below the constriction.

*Discussion.* In rabbits with sympathectomy and aortic constriction the denervated aorta was subjected to full systolic thrust above the constriction, but this force was considerably damped below that area. Page(2) suggested that atherogenesis is due to accumulation of substances filtered from plasma through the intima by the lateral pressure. Therefore, in this study decreased lateral pressure below the level of aortic constriction may explain diminished atherogenesis as a re-

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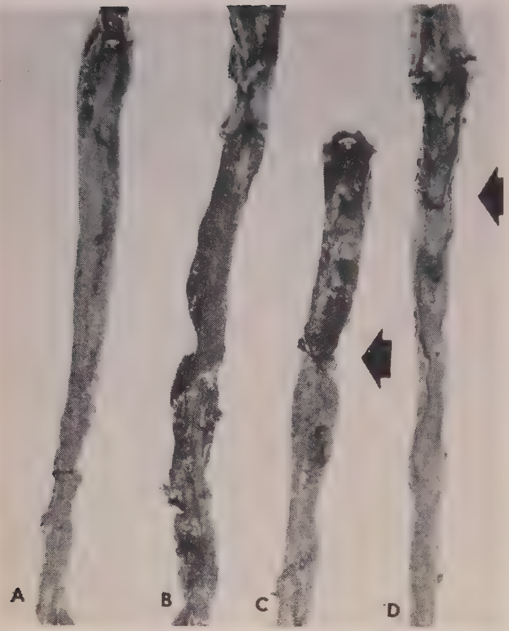

FIG. 1. (A) Aorta of non-operated rabbit on high cholesterol diet; (B) aorta of sympathectomized rabbit on high cholesterol diet; (C & D) aortas of sympathectomized rabbits with aortic constriction on high cholesterol diet. (Arrow points to site of constriction.)

sult of decreased filtration of lipemic plasma through the intima.

The mechanism by which sympathectomy increases the severity of abdominal atherosclerosis in rabbits is not clear. Murphy(1) postulated that it is the combination of 2 factors, first, decrease in peripheral resistance and consequent increase in blood flow, and second, weakening of muscular wall of the artery due to paralysis of smooth muscle fibers. It is also possible that the metabolic transport capacity of the normal rabbit lumbar aorta is better able to handle the filtered substances than is the denervated vessel.

*Summary and conclusions.* Rabbits subjected to bilateral lumbar sympathectomy and fed a high cholesterol diet developed severe atherosclerosis in the lumbar aorta and iliac arteries. When an aortic constriction was concomitantly produced, atherogenesis distal to the constriction was diminished. This was accompanied by a decreased systolic and diastolic pressure, a diminished pulse pressure, and absence of visible aortic pulsation below the constriction.

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## Effect of Bovine Fraction VI on Thyroid Function in the Rat. (24421)

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When normal human serum is labeled with tracer dose of $I^{131}$ thyroxine and electrophoresis carried out on paper strips at pH 8.6 in a barbital buffer $\mu = 0.075$, radioactivity will be located in region of the $\alpha$-globulins(1), provided the thyroxine level is not too high. Robbins, *et al.*(2) have demonstrated that this is due to the presence at pH 4.5, of a thyroxine-binding protein (TBP) with an isoelectric point close to pH 4. Freinkel, *et al.*(3) have demonstrated the presence of such a TBP in human serum fraction VI but confining their efforts to fraction IV-6 have isolated in very low yields a TBP whose binding capacity appeared to be easily lost(4). We have chosen to work with fraction VI since this fraction, according to the Cohn scheme of fractionation, is richest in glycoprotein.

*Method.* Bovine fraction VI (BFr VI) isolated according to an extension of Cohn method 10(5) by Pentex Corp. was labeled with $I^{131}$ thyroxine, obtained chromatographically pure from Abbott Laboratories and electrophoresis carried out in duplicate on paper strips under conditions described above. The Spinco Model R paper electrophoresis apparatus was used. In the experiments recorded here, 20 $\lambda$ of labeled protein solution

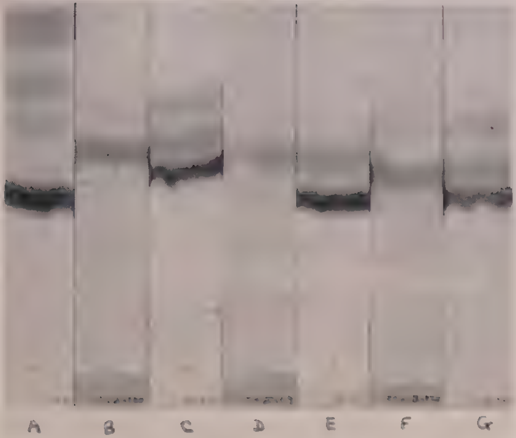
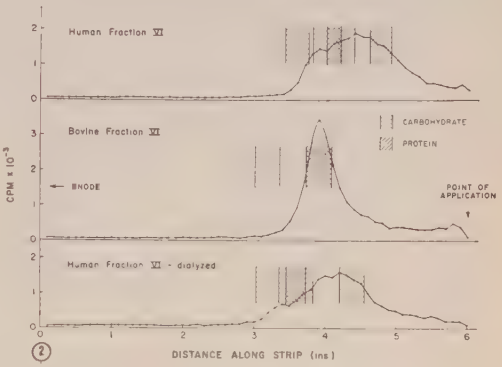


FIG. 1. Electrophoretic patterns of human and bovine fractions VI. A—normal human serum, bromphenol blue stain; B—human serum fraction VI, Köiw's stain; C—human serum fraction VI, bromphenol blue stain; D—bovine serum fraction VI, Köiw's stain; E—bovine serum fraction VI, bromphenol blue stain; F—human serum fraction VI-dialysed, Köiw's stain; G—human serum fraction VI-dialysed, bromphenol blue stain.

was applied to the paper, the concentration of protein being 2.6% for BFr VI and 2.8% for human fraction VI (HFr VI) dialyzed and undialyzed. In all these cases, 0.73 $\mu$c of $I^{131}$ thyroxine was included in the 20 $\lambda$ applied to the paper, corresponding to $5.2 \times 10^{-2}$ $\gamma$ of thyroxine. Exactly similar results have been obtained in which the protein concentration was 5.2%, thyroxine activity 0.69 $\mu$c corresponding to $2.40 \times 10^{-2}$ $\gamma$ thyroxine. Considering that only 10 g BFr VI can be obtained from 13 liters of plasma, these thyroxine levels are well below saturation for the TBP. One paper strip was then scanned for radioactivity and stained with bromphenol blue to indicate protein bands, and the duplicate strip was stained with Köiw's stain(6) to indicate carbohydrate bands. Fig. 1 and 2 show that under pH and ionic strength conditions used, 2 protein bands appeared, one of which represents a glycoprotein. Moreover, in this sample of bovine fraction VI, essentially all radioactivity was located in a narrow band coincidental with the glycoprotein band. This result has been reproduced many times with this same bovine fraction VI, and similar results were obtained with samples from other batches. HFr VI, also obtained from Pentex Corp., by method 10 of Cohn, examined electrophoretically under the same conditions always showed a less clear picture. Three protein bands were evident (Fig. 1, 2), one of which was characteristic of glycoprotein, while thyroxine seemed to spread itself out far more, as indicated by the radioactivity scan, than was the case of the BFr VI. The effect of BFr VI on thyroid function in the rat has been investigated. The properties studied were 1) 24-hour thyroid uptake of iodide ion, 2) 24-hour conversion ratio of inorganic to protein-bound iodine, and 3) body retention of iodine. Four- to 5-month-old female rats of Sprague-Dawley strain were given intravenously in the tail, various doses of BFr VI dissolved in normal saline solution. When multiple doses were given, these were at 24-hour intervals. Twenty-four hours prior to withdrawal of blood by cardiac puncture, they were injected with 5 $\mu$c of carrier-free $I^{131}$ iodide, simultaneously with the last dose of Fr VI. After 4-6 ml of blood had been drawn, the rats were



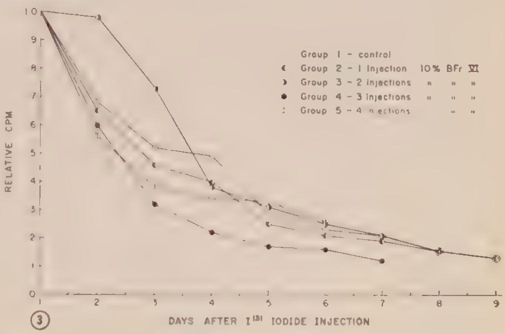


FIG. 2. Radioactivity scans of electrophoretic strips.

FIG. 3. Effect of bovine fraction VI on body retention of iodine in 5 groups of rats. The error (mean deviation from mean) in each point is, for the most part, <10% and >20%.

TABLE I. Depression of Rat Thyroid Activity by Intravenous Injections of Bovine Fraction VI.

| Treatment | Dose | No. of rats | C.R. | Thyroid, cpm × $10^{-3}$ | Thyroid count / Serum count |
|---|---|---|---|---|---|
| None | None | 6 | 35.9 ± 2.7 | 295 ± 88 | 50.5 |
| " | " | 8 | 32.8 ± .7 | 146 ± 39 | 50.1 |
| " | " | 10 | 53.1 ± 5.0 | 164 ± 23 | 61.4 |
| BSA* | 1 × .4 ml 10% | 4 | 37.4 ± 4.9 | 118 ± 20 | 76.0 |
| " | 4 × .5 20 | 7 | 41.3 ± 5.5 | 305 ± 55 | 55.6 |
| BFr VI | 1 × .5 20 | 4 | 26.1 ± 2.7 | 36.9 ± 12.6 | 13.9 |
| " | 4 × .5 20 | 13 | 16.1 ± 5.4 | 263 ± 44 | 41.8 |
| None† | None | 5 | 27.9 ± 5.5 | | |
| BSA† | 1 × .2 ml 10% | 10 | 26.2 ± 9.2 | | |
| BFr VI† | 1 × .2 " | 9 | 30.4 ± 6.7 | | |
| BSA‡ | 1 × .4 ml 10% | 4 | 63.2 ± 5.3 | 78.5 ± 3.9 | 88.2 |
| BFr VI§ | 1 ×1.0 " | 5 | 14.6 ± 9.3 | 154 ± 20 | 10.8 |
| " | 1 ×1.0 " | 4 | 33.1 ± 8.7 | 62.8 ± 15.3 | 12.1 |

4 × .5 ml 20% indicates 4 doses of .5 ml of 20% solution given at 24-hr intervals. Errors are mean deviations from mean.

* Some serums were pooled. † 7-wk-old, female Sprague-Dawley rats. ‡ 48-hr conversion ratio and thyroid count. § Results unreliable due to difficulty of inj. 1 ml into tail vein.

killed by overdose of ether, and their thyroid glands, together with a small section of trachea were removed and counted in a well-type scintillation counter. Serum was separated from coagulated blood after clot and cells had been centrifuged, and from 2-3 ml of serum were counted in the scintillation counter. From this counted volume of serum, the proteins were precipitated by addition of excess 10% trichloracetic acid, (TCA) centrifuged, and washed thoroughly with small volumes of 10% TCA, this procedure being repeated twice. The protein precipitate was then counted in the scintillation counter, the ratio of counts/minute of the protein to that of serum being the conversion ratio (C.R.). Two control groups of rats were treated similarly: the first group received only $I^{131}$ iodide; the second crystalline bovine serum albumin (BSA) (Armour) in exactly similar doses to those receiving bovine fraction VI. The results are summarized in Table I. In body retention experiments, 5 groups of 5 rats were given increasing doses of bovine fraction VI intravenously in the tail, followed by a 5-$\mu$c tracer dose of $I^{131}$ iodide. The whole rat was then counted on successive days in a large well-type scintillation counter, and the mean value for each group, relative to the count obtained one day after $I^{131}$ iodide injection was taken as a measure of body retention of iodine. Group 1 received no bovine fraction VI and therefore acted as control; the remaining 4 groups received, respectively 1, 2, 3 and 4 injections of 0.4 ml of 10% bovine fraction VI, given at 24-hour intervals, the last dose given simultaneously with $I^{131}$ iodide.

*Results.* The results are shown in Fig. 3. No significant differences were observed in renal clearance of $I^{131}$ between treated and untreated rats.

Table I shows that the conversion ratio for rats treated with bovine serum albumin does not differ significantly from that of untreated controls, whereas a single dose of 0.5 ml 20% BFr VI resulted in a significant fall, and, 4 times this dose resulted in a marked drop in conversion ratio. Thyroid uptake measurements, as indicated by ratios of thyroid to serum counts, suggest that uptake is initially inhibited by comparatively small doses of BFr VI but unaffected by BSA. However the Table suggests the possibility that prolonged doses of BFr VI allow the thyroid gland to recover its capacity to trap iodide ion.

*Conclusions.* Bovine fraction VI has a component, possibly a glycoprotein, capable of binding thyroxine to the exclusion of other components: moreover, bovine fraction VI markedly inhibits conversion of inorganic iodide ion to organic protein bound iodine in the rat. This inhibition can be attributed neither to decreased body retention of iodine nor to foreign protein reaction, but seems to take place either at organification of the iodine stage or at subsequent stages that lead to secretion of thyroid hormone. It has been shown that thyroxine or desiccated thyroid gland(7) as well as triiodothyronine(8) given either orally or intravenously produce an immediate depression in thyroidal uptake of iodide ion but leaves the level of protein-bound iodine (PBI) unchanged. The depressive effect lasts beyond removal of exogenous thyroxine. It does not therefore seem probable that the depressed conversion ratio is a result of thyroxine or triiodothyronine bound to the glycoprotein component of fraction VI.* We are currently carrying out fractionation procedures to separate or at least concentrate this factor.

*Summary.* Serum fraction VI from a bovine source showed on paper electrophoretic separation the presence of a glycoprotein band capable of binding $I^{131}$ labeled thyroxine to the exclusion of other proteins present. On being given intravenously in the tail of rats, bovine fraction VI gave a marked depression in conversion ratio of inorganic to protein-bound iodine. This effect was shown to be due neither to a foreign protein reaction nor to a decreased body retention of iodine. The uptake of iodide ion by the thyroid gland did not appear to be the cause of this depressed conversion ratio.

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* In fact, we have examined samples of BFrVI for iodine content and have found the complete absence of iodine. These results have been presented for publication in this Journal.

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## Thyroid Depressant Activity in Bovine Fraction VI. (24422)

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We reported(1) the presence of a thyroxine-binding protein (TBP) in bovine fraction VI (BFrVI) which was identified as a glycoprotein. It was also reported that BFrVI has a depressant effect on thyroid activity, as measured by the conversion ratio, when given intravenously to the rat. It was suggested that this effect might be attributable to thyroxine(2) accumulated by the TBP. We have since obtained many samples of BFrVI which the present paper shows have markedly higher thyroid depressant activity than those previously described, and have demonstrated that the activity is not due to presence of thyroxine nor to versene or heavy metals which may be present as impurities. The method for assaying the BFrVI for thyroid activity has been described(1). Sprague-Dawley female rats, weighing 250 to 300 g each, were used unless otherwise stated. Table I gives evidence of the markedly increased thyroid depressant activity of 2 samples of the same lot of BFrVI,* a single dose of 0.3 ml of a 20% fraction VI solution giving a conversion ratio of 16.8 compared with a value of 16.1 ± 5.4 previously obtained with 4 doses of 0.5 ml of a 20% solution(1). *Determination of*

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* Obtained from Pentex Corp.

TABLE I. Comparison of Different Samples of Bovine Fraction VI.

| Sample No. | Dose (ml 20%) | C.R. |
|---|---|---|
| 3-8205 | .5 (2) | 11.1 ± .2 |
| | .3 (1) | 16.8 |
| 2-8205 | .5 (4) | 9.2 ± .4 |
| | none (2) | 32.1 ± .2 |
| | " (3) | 32.7 ±2.7 |

Numbers in parentheses represent No. of rats used. C.R. represents Conversion Ratio defined as ratio of cpm on precipitated protein from 3 ml serum to that on 3 ml serum, 24 hr after intrav. inj. of 5 $\mu$c $I^{131}$ iodide (carrier-free)(1).

The errors given in this and subsequent tables represent mean deviations from the means.

*protein-bound iodine.* To examine the possibility that thyroxine was present in these protein fractions, we determined the protein-bound iodine (PBI) by a method described by Moran(3). All water used in the determination and for washing glass apparatus was distilled prior to passing through an anion-cation exchange resin. Optical density measurements were made with 1-cm cuvettes on a Beckman Spectrophotometer, Model DU, at 420 m$\mu$. Standard iodide solutions were used to establish a calibration curve in terms of PBI units, *i.e.*, $\gamma$ per 100 ml serum. Such a curve is shown in Fig. 1, giving some idea as to reproducibility. A standard sample of Iodotrol† was examined for its iodine content, and a sample of the same lot number returned to the Hormone Chemistry Laboratory for PBI determination. The recipients in the Hormone Chemistry Laboratory were unaware of the nature of the sample. At this time, 2 samples of the BFrVI, lot No. 8205, the first, 60 mg, the second, 600 mg, were examined for iodine content, and the plasma from 6 normal rats was examined similarly for comparison. The results are shown in Table II. We have taken the mean of the optical densities in 16 determinations on reagent blanks as most meaningful, since occasionally the apparatus on standing becomes slightly contaminated and this is reflected in the reagent blank. This mean is 1.32 ± 0.06 and this figure is used to determine PBI given in the last column; however, figures given in parentheses are the PBI determined using the reagent blank figures of column 3. The number returned to us from the Hormone Chemistry Laboratory was 6.3 ± 0.2 $\gamma$ %. It is clear that the PBI determinations are valid, and that there appears to be no iodine in BFrVI. Schmidt(4) reports 10 g of protein in human fraction VI from 13 liters of plasma; we may therefore estimate that 600 mg of BFrVI represents 780 ml serum. The usual volume is 3 ml of serum for PBI determination by the Moran method.

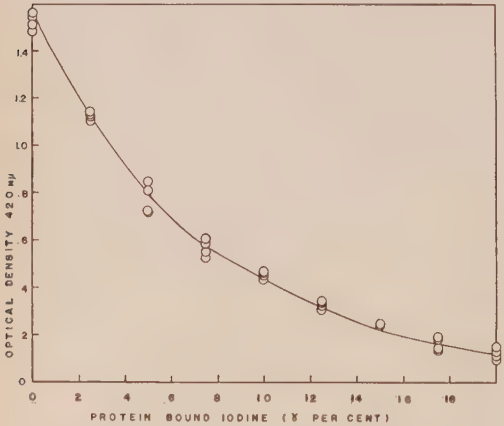

FIG. 1. Reproducibility of standard curve in determination of iodide by method of Moran(3).

TABLE II. Determination of Protein-Bound Iodine.

| Sample | OD (420 m$\mu$) Unknown | OD (420 m$\mu$) Reagent blank | PBI ($\gamma$ %)* |
|---|---|---|---|
| 3 ml Iodotrol | .568, .570 | 1.19, 1.25 | 6.4 (5.85) |
| BFr VI | | | |
| 60 mg | 1.35, 1.35 | 1.40, 1.35 | 0 |
| 600 mg | 1.29, 1.29 | 1.38, 1.39 | .10 ( .50) |
| 3 ml rat serum | | | |
| 1 | .908, .925 | 1.34, 1.37 | 2.6 (2.8 ) |
| 2 | .740, .735 | 1.38, 1.39 | 4.2 (4.55) |
| 3 | .825, .805 | 1.31, 1.28 | 3.5 (3.4 ) |
| 4 | .900, .905 | 1.33, 1.35 | 2.65 (2.8 ) |
| 5 | .860, .845 | 1.18, 1.16 | 3.15 (2.3 ) |
| 6 | .890, .805 | | 2.80 |

* Corrected for reagent blank.

*Results. Effect of sodium versenate.* In preparation of BFrVI(4), the proteins are precipitated in the presence of excess barium ions and are subsequently removed by dialysis in presence of versenate over a period of sev-

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† All glass apparatus and all chemical reagents specially prepared to be iodine-free and Iodotrol, a synthetic plasma preparation of known iodine content, supplied by Hormone Chemistry Lab.

TABLE III. Effect of Neutralized Versene and of Barium Ions on Thyroid Function.

| Dose | C.R. |
|---|---|
| .10 mg Versene | 30.6 |
| .25 *Idem* | 31.1 |
| 1.0 " | 31.9 |
| 2.0 " | 38.7 |
| 5.0 " | 30.1 |
| .1 ml 20% (4) | 37.6 ± 5.9 |
| .2 " " (3) | 39.0 ± 5.4 |
| Control (11)* | 37.7 ± 4.3 |

* Control was performed on untreated rats of same strain and age as exp. rats. Numbers in parentheses represent No. of rats used.

eral days. To examine the possibility that some versenate had remained in the lyophilized fraction VI, which might account for the results obtained, a series of rats was given injections in the tail vein of 0.5 ml of a solution of sodium versenate at pH 7.4 so as to contain various quantities of the chelating agent. Simultaneously, they were given injections of 5 μc of $I^{131}$ iodide, and a 24-hr conversion ratio was obtained. The rats used were 450- to 500-g Holtzman males. Results are given in Table III.

A normal conversion ratio determined on 11 of these male rats gave a value of 37.7 ± 4.3. It seems possible that the data may indicate a slightly depressed conversion ratio, but this could not account for the marked effects of the BFrVI. One-tenth mg has about the same effect as 5.0 mg of versene. This is not surprising since it has been shown that from 60 to 70% of the versene given intravenously to the rat is excreted in the urine in 2 hours and from 90 to 95% in 6 hours(5). Moreover, 5 mg would represent 5% by weight of the usual dose of BFrVI (0.5 ml 20%) given to the rat and one would have to reach an inordinately high versene content to obtain a conversion ratio of 10%.

*Effect of heavy metal ions.* We have received from Pentex Corp. samples of BFrVI (lot No. 8206), which are insoluble in a normal saline solution, unlike the usual samples which are readily soluble. The insoluble samples, however, are readily soluble in a neutralized versene solution (1%). We assume, therefore, that insolubility is due to presence of heavy metal ions, namely, barium. This suggested that low concentrations of such ions may even be present in the soluble BFrVI samples and that they may affect thyroid function in the rat. To test this possibility, 7 400 to 500 g Holtzman male rats were each given from 0.1 to 0.4 ml of a 20% suspension of an insoluble sample of BFrVI *via* the tail vein simultaneously with 5 μc of $I^{131}$ iodide. The one given 0.4 ml died in a matter of seconds, that given 0.3 ml died within 2 minutes, and 2 others were dead in a matter of hours. In a group of 5 rats given 0.2 ml, 2 were dead by the end of 24 hrs, and in a group of 5 rats given 0.1 ml, one died by the end of 24 hrs. Those surviving at the end of 24 hrs were examined for thyroid activity, and results are shown in Table III.

It is obvious that barium ions do not account for the depressed conversion ratio found on treatment with BFrVI. To demonstrate the activity of this insoluble material, 2 g were dissolved in 25 ml of a 3% neutralized versene solution at pH 7.2 and dialyzed against running tap water for a total of 10 hr and against distilled water for 80 hr, at which time optical density of the dialysate was zero, down to a wavelength of 240 mμ. This dialyzed BFrVI solution was approximately 8% in protein and could be assumed free of both heavy metal ions and versene. Intravenous injections of 0.5 ml quantities of this solution (approximately one-third of normal dose) were given to groups of Holtzman male and Sprague-Dawley female rats for comparison, as previously described. Results are shown in Table IV. In addition, a separate com-

TABLE IV. Comparison of Holtzman Male and Sprague-Dawley Female Strains.

| Strain | Dose | C.R. |
|---|---|---|
| Holtzman ♂ (4) | .5 ml 8% solution dialyzed | 35.1 ± 2.8 |
| Sprague-Dawley ♀ (6) | *Idem* | 19.0 ± 4.3 |
| Holtzman ♂ (3) | .5 ml 20% solution untreated and undialyzed | 19.3 ±13.7 |
| Sprague-Dawley ♀ (3) | *Idem* | 11.4 ± 5.2 |
| *Normal C.R.* | | |
| Sprague-Dawley ♀ (29) | | 39.6 ± 3.4 |
| Holtzman ♂ (11) | | 37.7 ± 4.3 |

Numbers in parentheses represent No. of rats used.

parison was made on these 2 strains of rats using a normally soluble and, therefore, untreated sample of fraction VI. These results are also shown in Table IV.

It appears that although the Holtzman male rat is a less sensitive and less reliable rat for this bio-assay, it does respond to a high enough dose of BFrVI. Moreover, there is no doubt that the sample of fraction VI, after treatment with versene and prolonged dialysis, does in fact show a depressant effect on thyroid activity, but in the dosage given, only by the more sensitive Sprague-Dawley female rat.

*Discussion.* The fact that BFrVI contains a TBP identifiable as a glyco-protein as well as a component showing thyroid depressant activity may be fortuitous. However, the TBP of Ingbar, Freinkel, and Dowling(6), obtained from serum fraction IV-6 was also identified as a glycoprotein and it may be that ability to bind thyroxine is a property of carbohydrate-containing proteins in general. Thyrotropic hormone (TSH) has been identified as a glycoprotein by 2 independent means(7,8), and there is considerable evidence available to show that TSH is inactivated after reaching the thyroid gland(9,10,11), or simply by acetylation(12). Moreover, this acetylated product acts as a TSH inhibitor. It is also known that there is present in the urine of hypothyroid patients(13) a TSH inhibitor that was shown to be a protein of low N content and could in many respects be likened to TSH. It was suggested that this inhibitor was in fact an inactivated TSH. The possibility exists that the thyroid depressant present in BFrVI is an inactivated TSH, acting as a TSH inhibitor, and that may be capable of binding thyroxine.

*Summary.* Some very active samples of BFrVI have shown marked ability to depress thyroid function as measured by the conversion ratio. Careful examination of these samples has shown that there is little or no iodine in the serum fraction, so the presence of thyroxine or any other iodine-containing calorigenic substance cannot be used to explain these results. The possibility that either versene or barium ions, both used in preparation of fraction VI, are responsible for this activity has been discounted. The higher sensitivity of the Sprague-Dawley female rat in comparison with the Holtzman male rat in this bio-assay has been demonstrated. Further studies are in progress attempting to elucidate the nature of this thyroid depressant in bovine fraction VI.

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# Effect of Irradiation on Some Portions of Head of Young Axolotl (*Siredon mexicanum*)*† (24423)

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In our previous reports concerning influence of x-ray irradiation upon development of the head(1,2), the great sensitivity of teeth and eyes was demonstrated. We are interested now in comparative sensitivity of various portions of the head of these animals.

*Materials and methods.* A total of 163 young axolotls (25-30 days after hatching) were used in this experiment. Forty-three animals served as untreated controls, 105 were irradiated with a dose of 3,000 r and one group of 15 animals was irradiated with a dose of 6,000 r. The conditions of irradiation were as follows: 100 kv, 30 ma, 0.5 mm Al filter, beryllium window tube; half value layer 1.25 mm Al; target object distance 18 cm. The animals were anesthetized before irradiation by placing them in an aqueous solution of M. S. 222 (Tricaine methanesulfonate), until they were anesthetized. During irradiation the animals were arranged in radial position within a circle on moistened filter paper. The posterior portions of the animals were covered with moist cotton. The nonirradiated portions were shielded with a lead covering with a round radiation localizer about 10 cm in diameter (Brunst, 3). Fixations were made using a saturated solution of bichloride of mercury with 5% acetic acid and in acetic mercuric chloride formalin (Stieve). 70 irradiated animals and 10 controls were examined histologically.

*Results.* Definite growth suppression of the irradiated portion of the head was observed 80-110 days after treatment. A dose of 3,000 r was lethal in all cases if the whole head was irradiated (all animals died within 6 months after treatment). However, if only the anterior portion of the head was irradiated with 3,000 r, all of the animals were not killed; some animals lived more than 20 months, when suppression of growth became especially obvious.

As a result of the disturbance of development, the visceral arches are exposed. In normal animals these are always covered. In most animals the eyes had disappeared or rudimentary eyes were covered by skin several months after irradiation.

Graphic reconstructions show that the most typical effect is suppression of development of the entire anterior portion of the head and complete disappearance of teeth. While growth of olfactory chambers is much suppressed, they never disappear completely. Owing to suppression of growth of the olfactory chambers, the anterior portion of the brain is reduced. Growth of the lower jaw is not only suppressed, but the whole anterior portion of the jaw has disappeared and as a result, the visceral (branchial) arches supporting the gills are shifted forward.

Comparison of the reconstruction of an animal on the day of treatment, 30 days after hatching, with an experimental animal 174 days after irradiation of the anterior portion of the head with 3,000 r, 204 days after hatching, shows that growth of the irradiated portion of the head is almost completely suppressed and that the anterior portion of the head has almost the same size as on the day of irradiation. The posterior portion of head of the same animal that was not irradiated is greatly enlarged. For example, the size of lymph nodes and branchial arches is 3-4 times larger than on the day of treatment.

*Cornea.* The normal cornea of axolotl consists of substantia propria and epithelium of cornea. The pigment cells and Leydig's cells that can be found in normal skin epithelium are absent in normal epithelium of cornea. Following irradiation, the whole cornea never disappeared. In some cases the substantia propria was almost normal in appearance and thickness; in other cases, it was 2 to 4 times thicker than normal. As a result of abnormal

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thickness or development of pigment cells the substantia propria lost their transparency in some cases. Rohrschneider(4) observed the same phenomenon in irradiated rabbit cornea. Damage of corneal epithelium was observed in 38% of all irradiated animals. In some cases the reduction of the number of layers of this epithelium was typical; in cases of extreme damage only 2 layers of cells were observed with simultaneous development of giant cells. In other cases, the epithelium was thicker than normal, but Leydig's cells, and giant cells, never present in normal epithelium, were found. In cases of the most severe corneal damage, observed in 30% of the irradiated animals, corneal epithelium disappeared completely and was replaced by typical skin epithelium with many Leydig's cells. The entire cornea was normal in 32% of the irradiated animals.

*Lens*. The normal lens of control animals has almost homogeneous structure with slight concentric layers. The lens is covered with a capsule, a cuticular membrane. Cracks of the lens are very typical. The formation of these cracks obviously is artefact, but at the same time is a response of definite type of lens structure to fixation. They are mostly observed in a definite direction—parallel to the direction of lens fibers. These cracks were observed consistently in the lens of non-irradiated animals, but they disappeared almost completely even after slight roentgen damage. In some cases, cracks appeared only in the central portion of irradiated lens, when the peripheral portion is entirely changed. Damage of the lens was observed in 56% of irradiated animals. Slight damage was observed in 5%, characterized by many typical granules, vacuoles, and spheres. Moderate damage was observed in 22%, characterized by great disturbances in all peripheral portions. More than half of the lens was completely transformed into a globular mass having various sizes of granules, vacuoles, and spheres. The central portion alone retained its original structure. Serious damage was observed in 29%, characterized by almost complete disappearance of normal lens substance as a result of dissolving of the lens fibers; only remnants of the lens were noted in most cases. The lens membrane, however, was the most resistant structure, although many small folds were typically found. In many cases, after total disappearance of the lens, a wrinkled lens membrane alone was found. A most significant observation was the presence of a great number of large cells in the body of the seriously damaged lens. These were wandering giant cells, macrophages, *i.e.* phagocytes destroying the substance of the irradiated lens. They partially phagocytized and partially dissolved the lens fibers, both these activities being typical of these cells. Cells are never found in the normal lens. The lens was entirely absent in 28% of the irradiated animals.

*Retina*. In the retina it is necessary to distinguish between damage of the rods and cones layer and damage of the other layers. According to our previous observations the rods and cones layer of the retina is the most sensitive. The sensitivity of this layer is almost the same as the sensitivity of the epithelium of the cornea. In 31% of all cases the rods and cones layer was completely absent, in 33% it was damaged. Serious damage was found in 9% of the animals, where only remnants of this layer were preserved; moderate damage in 15%, where about half of the layer was badly damaged; partial damage in 9%, where only portions of the layer were partially damaged. Finally, this layer was normal in appearance in 36% of the irradiated animals. Retinal layers other than the rods and cones are much more resistant to irradiation. Complete absence of retina was not observed and in only 6% of the irradiated animals was damage of all retinal layers so severe that only small rudiments of retina were noted. In 4% about half of the retinal layers were destroyed and in 6% only portions of the layers were damaged. In most cases (84%) of the retinal layers appeared entirely normal.

*Teeth*. Teeth were entirely absent in 89% of irradiated animals. However, teeth were still found in animals fixed within 30 days after irradiation, that is, during the latent period for the disappearance of teeth. With these exceptions, teeth were absent in 100% of the animals. In the head of the axolotl teeth may be the structure most sensitive to roentgen irradiation.

*Lymph nodes.* In normal axolotls several pairs of large lymph nodes are situated in the boundary region between the head and trunk. After irradiation of the whole head, lymph nodes disappeared in 100% of the animals. After irradiation of only the anterior portion of the head, lymph nodes appeared normal in 95% of the animals; in the remaining 5% they were slightly abnormal and, being in the boundary zone of the irradiated area, obviously had been partially irradiated. Such partial irradiation may have been the result of slight movement of the animals during treatment, or even of slight error in their positioning for treatment—if they had been placed too near the center of the irradiated field, the posterior portion of the head, including the region of the lymph nodes, would have received some irradiation. The lymph nodes, as well as other types of lymphoid tissue are highly sensitive to irradiation.

*Gills.* In evaluation of damage to gill epithelium it is important to consider time of fixation. When gills were fixed during the primary acute reaction (20-60 days postirradiation) severe damage is usually found, as evidenced by the formation of giant cells. When gills were fixed later, the appearance of gill epithelium was almost normal. In such epithelium it is often possible to see the Leydig's cells which are typical of old skin epithelium but not gill epithelium. However, in some cases the epithelium of the gills appeared normal even within 20-60 days after the animals were irradiated. In all probability, such gills themselves were not irradiated. It is obvious that local irradiation of even the whole head of the axolotl will not result in consistent irradiation of the gills, because of the location of the gills at the boundary between head and trunk.

*Olfactory chambers.* The olfactory chambers are large organs taking up considerable space in the anterior portion of the head of the normal axolotl. Normal olfactory epithelium has a typical structure and many sensitive olfactory buds which are modifications of the sensitive skin buds distributed in some regions of the skin. Normal olfactory epithelium appears distinctly different from normal skin, and Leydig's cells are not found in it. Normal olfactory epithelium was never found after irradiation. However, in 17% of the irradiated animals the epithelium was only slightly damaged; typical orientation of olfactory cells disappeared. With one exception, such slightly damaged epithelium was discovered only after early fixation (13-36 days postirradiation). This suggests that olfactory epithelium reacts with comparative slowness to irradiation. All late fixations give a clear picture of damage of the olfactory epithelium. In 28% of these cases the olfactory epithelium disappeared completely and was replaced by typical skin epithelium. In 55% the olfactory epithelium had not disappeared, but was greatly damaged; cells similar to those of olfactory epithelium were found but without any special structures. Sensitive olfactory buds disappeared and the distribution of cells in the epithelial layer was completely abnormal. We conclude that olfactory epithelium is one of the tissues of the head most sensitive to irradiation.

*Discussion.* With respect to sensitivity to irradiation, tissues and organs of the head may be arranged in 5 groups: (I) Teeth and lymph nodes (disappeared completely in 100% of animals). (II) Olfactory epithelium (disappeared in 28%, damaged in 55%, slightly damaged in 17%), and the lens (disappeared in 28%, damaged in 56%, normal in 16%. (III) Epithelium of the cornea (disappeared in 30%, damaged in 38%, normal in 32%), rods and cones of the retina (disappeared in 31%, damaged in 33%, and normal in 36%), and the gills. (IV) Other layers of retina (damaged in 16%, normal in 84%). (V) Pigment cells of choroid, and other tissues of the head (comparatively resistant, no apparent damage after irradiation with 3,000-6,000 r under our conditions).

Characteristic damage was observed in several organs: specialized but not completely differentiated tissues destroyed by x-ray irradiation were never restored. These tissues include oral epithelium forming enamel organs of the teeth, olfactory epithelium of the nasal chambers, corneal epithelium, and possibly, gill epithelium. In all of these tissues the specialized epithelium which had disappeared completely was replaced by skin epithelium. A peculiarity of skin epithelium is the presence of Leydig's cells, never observed in differentiated epitheliums of enamel organs, olfactory chambers, cornea or gills. Therefore, specialized but not completely differentiated epithelium must be more sensitive to irradiation than skin epithelium.

*Summary.* 1) Definite suppression of growth of treated portion of the head was observed 80-110 days after irradiation with 3,000 or 6,000 r. In irradiated portions: teeth disappeared completely, growth of olfactory chambers suppressed greatly, but they never disappeared completely, anterior portion of the lower jaw had disappeared, visceral arches all shifted in rostral direction, development of the eyes was suppressed. According to sensitivity, it is possible to distribute the tissues and organs among 5 groups. (I) The teeth and lymph nodes (disappeared completely in 100% of animals). (II) Olfactory epithelium and the lens (disappeared in 28%, damaged in 55% and normal in 17%). (III) Epithelium of the cornea (disappeared in 30%, damaged in 38% and normal in 32%) and the rods and cones layer of the retina (disappeared in 31%, damaged in 33% and normal in 36%). The gills. (IV) Other layers of retina (damaged in 16% and normal in 84%). (V) Pigment cells of choroid and other tissues of the head, which are comparatively resistant and did not show any clear signs of damage. 2) Specialized but not completely differentiated tissues can be destroyed by X-ray irradiation and after this never restored, being replaced by typical skin epithelium with Leydig's cells. This was observed in oral epithelium, olfactory epithelium and epithelium of the cornea.

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## Myasthenia Gravis III: Lack of Inhibitory Action of Fractionated Thymus Extracts.* (24424)

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Several investigators(1-5) have studied the effects of thymus extracts on nerve muscle preparations from various animals. However, the reported neuro-muscular blocking activity of isolated frog sartorius muscle by acetone extracts of thymus glands from myasthenic patients(5), was probably due to large amounts of potassium in the extracts(6). The present study was undertaken to assay neuromuscular blocking activity of fractionated thymus extracts free of excess potassium.

*Methods.* The specimen studied was a surgically resected thymoma from a 30-year-old woman with myasthenia gravis. The tumor was roughly H-shaped and weighed 48.5 g. One lobe measured 14 cm in length and contained a 5 x 7.5 x 3 cm mass covered by a firm fibrous capsule. The other lobe measured 9 x 1.5 x 1 cm. The cut surface of the tumor showed lobules of soft yellow-white tissue separated by firm fibrous trabeculae. Microscopic examination of several blocks fixed in Helly's fluid and stained with hematoxylin and eosin showed a solid tumor composed of almost equal proportions of lymphocytic and epithelial cells. Used as a control was a sample of gastrocnemius muscle obtained at autopsy from a patient who died of cardiac disease. These tissues were extracted and fractionated† by the low temperature, low

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salt, ethanol procedure of Schmid, Rosa and MacNair(7). When applied to human plasma, 6 fractions are obtained. Fibrinogen (I), $\gamma$ globulins (II), $\alpha$ and $\beta$ lipoproteins (IV and III), $\alpha_2$ mucoproteins (IV), albumen (V), and $\alpha_1$ and $\alpha_2$ glycoproteins (VI) are separated. When the thymoma was fractionated according to this method, 6 fractions were obtained. The bulk of the material appeared in fractions I (175 mg), III (830 mg) and IV (990 mg). These fractions were not specifically analyzed. The skeletal muscle sample weighed 283.4 g and yielded 2 major fractions, one containing fractions I, II, and III (1269.4 mg) and the other, IV and V (6700 mg). Each fraction from the thymoma and from control skeletal muscle was tested on the isolated frog sartorius preparation in concentrations of 1 mg/ml of the lyophilized fraction in frog Ringer according to the method previously described(6). Fractions, with exception of thymoma fractions V and VI, were also dialyzed against distilled water at 4°C for 48 hours, lyophilized and tested for neuromuscular blocking activity. Several control runs with frog Ringer in place of the test extracts were also carried out to determine changes in responsiveness of isolated test muscle. Time for 50% inhibition/mg (T50) was computed for each fraction. Each fraction was tested on at least 6 frog muscle preparations. Potassium content of aliquots from each fraction was determined spectrophotometrically (Table I).

*Results.* Table I presents the results obtained.

*Comment.* The data show slight to moderate inhibition by all the thymoma and skeletal muscle fractions tested. None of the thymoma fractions showed the dramatic blocking activity that might be expected if an active principle had been isolated. Potassium content of all the extracts was in the range compatible with normal neuro-muscular function. Thus, by this technic no blocking activity was found.

TABLE I. Activity of Thymoma and Skeletal Muscle Fractions.

| Thymoma fraction | T50 min. | Td 50 | (K) meq/l* |
|---|---|---|---|
| I | 5.8 | 10.0 | 2.2 |
| III | 8.3 | 7.2 | 1.9, 2.6 |
| IV | 5.9 | 4.5 | 2.1 |
| V | 6.1 | | 1.9 |
| VI | 7.0 | | 2.5 |
| Skeletal muscle | | | |
| I, II, III | 6.5 | 6.9 | 1.7 |
| IV, V, VI | 7.8 | 7.5 | 4.9 |
| Frog Ringer | >15 | | 2.0 |

T50 = time (min.) for 50% inhibition of muscle contractions—undialysed fractions.

Td 50 = dialysed and lyophilized fractions.

* (K) of undialysed fractions.

*Summary.* Fractionated extracts from a thymoma surgically removed from a patient with myasthenia gravis when tested on an isolated sartorius nerve-muscle preparation failed to show significant inhibitory activity of any of the fractions when compared to similarly extracted normal skeletal muscle.

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# Neonatal Immunity. II. Poliocidal Effects of Human Amniotic Fluids.*†

(24425)

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The search for antibodies in body fluids and an interest in neonatal immunity has led to a search for antibody to the 3 polioviruses in amniotic fluid of the human subject at term. Although amniotic fluid is of vast importance to development of the fetus, information regarding its composition is scant. Data are available on total protein content and on concentration of various electrolytes(1). Little is known of the amounts of various blood proteins present such as gamma globulin, serum albumin, other globulins and fibrinogen. Work by Brambell and his colleagues(2) has shown that in the rabbit, antibodies appear in amniotic fluid after injection into maternal circulation. They also demonstrated the subsequent passage to and concentration of these antibodies in the stomach of the fetal rabbit, and suggested that it is conceivable that antibodies in the human newborn might come by way of the amniotic fluid. The steps might be as follows: swallowing, and concentration of fluid in the stomach and passage through stomach wall into the peripheral circulation. Concentration of proteins in amniotic fluid taken at birth has been reported(1) to vary from 0 to 1.5 g/100 ml of fluid, average 0.53 g. Quantitative data for gamma globulin content of amniotic fluid taken at term are at present not available. Amniotic fluid has been reported to be bacteriocidal(3). The nature of activity has not been established. This paper is a report on the presence of antibodies to the 3 poliovirus types in amniotic fluids of mothers who delivered normally at term.

*Methods.* Amniotic fluids (AF) free of blood were collected from women with unruptured membranes who delivered normally at term. One specimen was collected from patient at Caesarean section. Mothers' (MB) and babies' cord bloods (CB) were also collected. Mothers were in general of a low economic status and included both polio vaccinated and non-vaccinated individuals. Most had a high level of antibody to the 3 poliovirus types. The mothers were of various races, ages and previous obstetric experience. The fluids on arrival at laboratory were centrifuged, and those containing sedimented blood cells were excluded from this study. Some fluids were titrated for neutralizing antibody both before and after heat inactivation at 56°C for 30 minutes, others only after inactivation. Neutralizing antibody titers of amniotic fluid specimens and corresponding sera of mother and infant were determined simultaneously by a modification of the suspended cell colorimetric test for neutralizing antibodies, using first passage monkey kidney cells. Amniotic fluids cleared by centrifugation were diluted in 2-fold dilutions starting with undiluted fluid to a dilution of 1:512. Maternal and infant cord sera were titrated by doubling dilutions starting with initial dilution of 1:4. To each dilution was added 100 $TCID_{50}$ of a single type of poliovirus. All specimens were titrated for neutralizing antibody to all 3 poliovirus types. After incubation of amniotic fluid-virus and serum-virus mixtures at room temperature for 1 hour the monkey kidney cells obtained from bottles seeded with trypsinized kidney tissue were added to each tube and incubated 7 days at 37°C. The diluent used was 2% calf serum in Hanks' balanced salt solution with 0.5% lactalbumin hydrolysate. The test was read by microscopic examination for presence or absence of cytopathogenicity. The titer was expressed as the reciprocal of the last dilution of serum or amniotic fluid which showed complete protection. Gamma globulin was determined in amniotic fluids as follows: amniotic fluids cleared of cellular debris were diluted in 4-fold steps

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TABLE I. Distribution of Poliomyelitis Antibodies in Amniotic Fluid (AF), Maternal Serum (MB) and Cord Serum (CB) at Term.

| | | | Type 1 | | | Type 2 | | | Type 3 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Patient | Age | P/G† | AF | MB | CB | AF | MB | CB | AF | MB | CB |
| | | | | | | No record of vaccination | | | | | |
| 1 | 16 | 1/1 | 64 | 1024 | 1024 | 256 | 4096 | 4096 | 0 | 32 | 32 |
| 2 | 16 | 1/1 | 0 | 64 | 32 | 0 | 128 | 16 | 0 | 64 | 16 |
| 3 | 22 | 2/2 | 1 | 64 | 64 | 64 | 2048 | 2048 | 4 | 256 | 256 |
| 4 | 24 | 4/4 | 4 | 512 | 256 | 2 | 128 | 128 | 2 | 128 | 128 |
| 5 | 25 | 4/5 | 0 | <4 | <4 | 4 | 512 | 512 | 0 | 64 | 64 |
| 6 | 26 | | 2 | 64 | 128 | 2 | 256 | 256 | 0 | 32 | 32 |
| 7 | 29 | 6/6 | 0 | 32 | 32 | 8 | 1024 | 512 | 0 | 128 | 128 |
| 8 | 35 | 3/3 | 0 | 128 | 128 | 2 | 256 | 256 | 0 | 64 | 64 |
| 9* | 37 | 2/3 | <4 | 32 | | 2 | 256 | | <4 | 64 | |
| 10 | 39 | 11/14 | 0 | 32 | 16 | 1 | 64 | 64 | 0 | 16 | 16 |
| 11 | | 8/9 | 0 | 128 | 128 | 16 | 512 | 512 | 1 | 128 | 128 |
| | | | | | | 1 ml polio vaccine | | | | | |
| 12 | 15 | 1/1 | 4 | 256 | 512 | 2 | 1024 | 512 | 2 | 512 | 256 |
| 13 | 19 | 2/2 | 4 | 1024 | 512 | 8 | 1024 | 512 | 8 | 2048 | 128 |
| 14 | 26 | 6/7 | 2 | 1024 | 64 | <2 | 1024 | 16 | <2 | 1024 | 16 |
| 15 | 29 | 4/4 | 0 | 128 | 64 | 8 | 2048 | 1024 | 8 | 1024 | 1024 |
| | | | | | | 2 ml polio vaccine | | | | | |
| 16 | 16 | 1/1 | <16 | <4 | <4 | <16 | <4 | <4 | <16 | <4 | <4 |
| 17 | 18 | 2/3 | 16 | 256 | 128 | 8 | 1024 | 512 | 16 | 512 | 128 |
| 18 | 22 | 3/4 | 16 | 512 | 512 | 2 | 128 | 128 | 0 | 4 | <4 |
| 19 | 25 | 4/4 | 4 | 1024 | 512 | | | | | | |
| 20 | 26 | 6/6 | 4 | 2048 | 1024 | 2 | 1024 | 1024 | 4 | 4096 | 4096 |
| 21 | 30 | 11/11 | 0 | 64 | 32 | 2 | 128 | 64 | 1 | 64 | 64 |
| 22 | 38 | 5/5 | 64 | 256 | 256 | 32 | 512 | 256 | 32 | 256 | 256 |

* Caesarian section.

† P/G = parity/gravidity.

with saline. To 0.6 ml of each dilution was added 0.1 ml of anti-human gamma globulin rabbit serum whose precipitin titer (antigen-dilution method) was 1:50,000. The tubes were incubated at 37°C for ½ hour and over night at 4°C. Precipitation or flocculation was recorded. In a single experiment gamma globulin was concentrated from amniotic fluid using ammonium sulfate. Saturated ammonium sulfate was added to cold amniotic fluid to final concentration of 50% saturation. The precipitate so obtained was dissolved in one-tenth the original amniotic fluid volume, a 10-fold concentration. After dialysis at 4°C for 48 hours this solution was tested for presence of gamma globulin by the method outlined above. This solution was then titrated for presence of neutralizing antibodies against the 3 poliovirus types simultaneously with the original amniotic fluid.

*Results.* Table I gives data for each of 23 cases. In general, the ratio of neutralizing antibody of amniotic fluid to that of mother's serum, for each virus type is a constant within the limits of experimental error (Table I). An ideal case for demonstrating this is specimen from patient 3 where the ratio of AF to MB is 1:64 for types 1 and 3 and 1:32 for type 2 poliovirus. In instances where the amniotic fluid titer is 0 (less than undiluted) this ratio could not be determined. Disparity between observed antibody levels in matched pairs of cord and maternal blood is occasionally observed (*e.g.* patients 13 and 14), as discussed previously(4).

Twelve amniotic fluids were tested for presence of gamma globulin by the immunological procedure discussed under methods: The 12 were positive for presence of gamma globulin in dilutions ranging from 1:16 to 1:1024.

The polio-neutralizing antibody titers expressed as dilution reciprocals obtained in the single gamma globulin concentration experiment are as follows:

| | Type 1 | Type 2 | Type 3 |
|---|---|---|---|
| Original amniotic fluid | 4 | 8 | 8 |
| 10 × concentrated amniotic fluid | 16 | 16 | 64 |

The concentrated solution was titrated for presence of gamma globulin and compared with original fluid as follows: The endpoint of original fluid was between a dilution of 1:16 and 1:64 whereas the endpoint using concentrated material was 1:160. The concentrated solution on this basis was not 10-fold as expected but, closer to the 5-fold as indicated by the polio neutralizing antibody titers.

*Discussion.* The finding of neutralization of 3 poliovirus serotypes by human amniotic fluid is not unexpected, but the question arises as to whether this poliocidal activity is due to antibody. The demonstration of gamma globulin in the fluid, and the result of the concentration experiment make it likely that this activity is due to an immune mechanism. On the basis of an experiment in which both heated and unheated amniotic fluid was titrated for neutralizing activity it can be said that there is present a heat labile factor besides the heat stable factor which is poliocidal. Whether this is an antibody cannot be determined from our data.

The second question concerns the source of the antibody. The fact that antibodies to the 3 polioviruses are in almost constant ratio to maternal serum, points to transmission of serum gamma globulin from her blood stream to the amniotic fluid. The mechanism by which this occurs has not been elucidated.

It must be borne in mind that the relationship in antibody content of amniotic fluid, cord blood, and maternal blood is not constant for all antibodies. For example, it is well established that complete Rh antibodies do not pass into fetal blood as readily as incomplete Rh antibodies(5) and antibodies against the O antigens of enteric bacteria not as readily as flagellar antibodies(6). In preliminary experiments, unpublished, (Neter) on several specimens described in this paper the presence of antibodies against various O antigens of enteric bacteria was determined. The procedures employed have been described(7,8). The results obtained using selected amniotic fluids are shown in Table II. It was found that antibodies to various enterobacterial antigens used in the test appeared in only 1 of 6 amniotic fluids. This patient #22 also had high poliovirus neutralizing antibodies to the 3 types in her AF. These antibodies were in the ratio of 1:4, 1:16 and 1:8 respectively for the 3 types. On the other hand the ratio of enterobacterial antibodies were 1:2, 1:4 for hemagglutinins and 1:4 and 1:8 for Coombs hemagglutinins.

TABLE II. Enterobacterial Antibodies in Human Amniotic Fluids (Neter, 5).

| Patient | Specimens source | Hemagglutinin titers (reciprocal) | | | Coombs hemagglutinin titers (reciprocal) | | |
|---|---|---|---|---|---|---|---|
| | | Sa | Sh | EC | Sa | Sh | EC |
| 1 | AF | — | — | — | — | — | — |
| | MB | 80 | 320 | 80 | 80 | 320 | 80 |
| | CB | — | — | — | 40 | 20 | 20 |
| 3 | AF | × | — | — | × | — | — |
| | MB | 40 | 20 | 10 | 40 | × | 10 |
| | CB | — | — | — | 20 | — | — |
| 4 | AF | — | — | — | — | — | — |
| | MB | 40 | 80 | 80 | × | 160 | 80 |
| | CB | × | × | × | × | × | × |
| 13 | AF | — | — | — | — | — | — |
| | MB | × | × | × | × | × | × |
| | CB | — | 40 | — | 80 | 80 | 20 |
| 20 | AF | — | — | — | — | — | — |
| | MB | 160 | 80 | 160 | 160 | 160 | 160 |
| | CB | — | — | — | 40 | 40 | 20 |
| 22 | AF | 40 | 10 | 20 | 40 | 10 | 20 |
| | MB | 80 | 40 | 80 | 160 | × | 160 |
| | CB | × | × | × | × | × | × |

AF = amniotic fluid; MB = maternal serum; CB = cord serum.

Sa = polyvalent *Salmonella* groups $A_1$, $C_2$, $D_1$, $E_1$. Sh = polyvalent *Shigella flexneri* 3, *boydii* 1, *sonnei*. EC = polyvalent *Escherichia coli* 111, 55, 26.

— = less than 10; × = not done.

Patient 1, whose poliomyelitis antibody levels in the amniotic fluid were high for types 1 and 2 with a ratio of AF to MB of 1:16, had no detectable antibodies to the enterobacterial antigens. If the passage from maternal circulation to amniotic fluid were due to simple diffusion the ratio of 1:16 should hold and there would have been expected antibodies of a titer 10 to 20 to the polyvalent Shigella antigens by both methods used.

Perhaps the most important question concerns the protective role of amniotic fluid neutralizing substance. Our experiments probably, however, rule out the thought that the antibody in the fetus comes only or to a major degree by way of the amniotic fluid. The good agreement between amounts of polio neutralizing antibody in maternal and infant sera is evidence against the role of amniotic fluid as a source of infant antibody. If the mechanism involved this fluid the discrepancies between the 2 circulations would be expected to be much greater than the data indicate. Furthermore, many of the amniotic fluids do not have detectable antibody to some of the poliovirus types and the cord serums have appreciable levels of antibody.

*Summary.* Amniotic fluid of humans at birth has been demonstrated to contain poliocidal substance, active against all 3 serotypes of virus. Evidence has been presented which indicates that the activity is associated with the gamma globulin present in the amniotic fluid. The poliocidal activity is believed to be specific antibody. The presence of these antibodies has no correlation as far as can be determined with the age, color, polio immunization status of the mother nor with her previous obstetrical history. The level of antibody in the amniotic fluid for the 3 polioviruses was in proportion to the levels in the blood serum and in general was demonstrable when the serum level was high. There is also in amniotic fluid an inhibitor to the 3 polioviruses which is heat labile. Its character has not been determined. Enterobacterial hemagglutinins have been demonstrated in 1 of 6 AF tested.

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## Porcine Kidney Cell Cultures for Propagation and Assay of Japanese Encephalitis Virus.* (24426)

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Observations in 1956 at U.S. Army 406th Medical General Laboratory near Tokyo, Japan showed that swine serve as important source of Japanese encephalitis (JE) virus for *Culex tritaeniorhynchus,* Japanese vector mosquito, (unpublished). This finding together with the knowledge that JE virus causes stillbirth in swine(1), stimulated inquiry into possible effects of JE virus upon swine tissues *in vitro*. The initial study employed cultures of swine kidney because the basic principles were known for preparation of primary cultures from renal tissues(2). This article reports that cells in primary cultures from porcine kidney (PK) are rapidly destroyed by each of 5 strains of JE virus and concurrently support virus multiplication. Cultures of swine kidney cells thus furnish a tissue culture system for assay and propagation of JE virus.

*Materials and methods. Viruses.* Four strains of JE virus were isolated in Japan during 1955 and 1956 from swine blood, Black-crowned Night Heron blood, human brain, and triturated mosquitoes (*Culex tritaeniorhynchus*) (Table I). The fifth virus was Nakayama strain from Dr. C. M. Eklund (USPHS Rocky Mountain Laboratory). Mouse brain suspensions (10% in 10-40% rabbit serum in Hanks' solution) and cultural fluids were stored in sealed glass ampules at −70°C for as long as 2 months before being assayed. Cultural fluids were kept above pH 7.2 by addition of 1.4% aqueous sodium bicarbonate solution to minimize inactivation of JE virus that occurs at lower pH's(3). Kid-

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neys were from fetuses to 18 inches in length (head to foot) from 59 swine of various breeds slaughtered at a local abattoir and from 16 specially bred miniature pigs(4) less than 8 months old.† Fetal kidneys were most satisfactory because kidneys from mature pigs yielded few viable cells with the methods employed and kidneys from young swine were difficult to obtain regularly. Renal cortex was minced to 1 mm diameter fragments and rinsed free of erythrocytes with Hanks' balanced salt solution (BSS) without sodium bicarbonate (RBSS). The fragments, in a 100 ml screw capped bottle, were covered by Difco 1:250 trypsin, 0.25% in BSS at pH 7.6, and stirred on a magnetic mixer at 5°C for 15-20 hours or until they disappeared. Cells were sedimented at 38 g for 5 minutes, rinsed 2 or 3 times with 10-20 volumes of RBSS and suspended in measured volume of swine serum, 20%, and BSS, 80% (SwS-20, BSS-80) with or without Difco yeastolate at 0.1% final concentration. Penicillin and streptomycin were incorporated at 50-100 $\mu$g/ml. Cells were counted under 200x magnification in a hemocytometer, the cell concentration adjusted with SwS-20, BSS-80 to $10^6$ cells/ml, and 0.5 or 1 ml ($5 \times 10^5$, or $10^6$ cells) inoculated into each 16 x 125 mm screw capped test tube. Cultures were incubated in stationary slanted position at 36-37°C for 2-4 days before medium was changed or supplemented by addition of 1 ml of SwS-20, BSS-80. After another 3-4 days of incubation either the sheets of epithelial cells were satisfactory for virus experiments or the medium was again changed and incubation continued for several additional days. *Cultivation of virus in PK cultures.* The medium was changed to 0.9 ml of a mixture of swine serum, 5% (or 20% in one experiment), yeast extract, 0.1% (optional), penicillin and streptomycin, 50-100 $\mu$g/ml, and maintenance solution(5). To initiate experiments, mouse brain suspensions of virus were inoculated, 0.1 ml/tube culture, while for subsequent passages, harvests of cultural fluid and cells were made from 2-6 cultures when greater than 80% of cells were destroyed. For growth curve experiments, fluid and cells were pooled from 3 cultures at selected time intervals regardless of CPE. Incubation was at 36-37°C in a stationary position. Virus was assayed usually simultaneously in mice and PK cultures by inoculating decimal dilutions, in 0.03 ml volume, intracranially into 5 or 6 3-4 week old weanling Swiss albino mice or in 0.1 ml volume into 2 or 3 PK cultures containing 0.9 ml of SwS-5, MS-95/culture. For the Nakayama strain 1 ml of diluted virus (in SwS-5, MS-95) was placed in tube cultures void of fluid medium. Mice were observed for 10 or 14 days and cells for 7 days before $LD_{50}$ and $TCD_{50}$ were calculated(6). Virus from cultures was identified by virus dilution neutralization tests in PK cultures and/or in mice using specific rabbit antiserum made either at 406th Medical General Laboratory against the Nakayama strain, or at the USPHS Rocky Mountain Laboratory (furnished by Dr. C. M. Eklund).

*Results.* Evidence that JE virus propagates in porcine kidney cells was obtained by 2 types of experiments: 1) passage of virus through a series of cultures, and 2) measurement of virus growth in a single set of cultures. The cytopathogenic effects (CPE) of virus became evident within 2-5 days after inoculation of PK cultures with each of 4 virus strains, either as mouse brain suspensions or harvests from PK cultures. With Nakayama strain, total cell destruction did not occur until 6-10 days after inoculation.

*Serial passage of JE virus in porcine kidney cultures.* Five strains of JE virus derived from different natural sources were inoculated as mouse brain suspensions into PK cultures, and subsequently passed serially 5-28 times (Table I). Each passage was made when 80% or more of cells were destroyed, *i.e.,* at 2-4 day intervals for recently isolated strains, and at 6-10 day intervals for high mouse-and egg-passaged Nakayama strain. Mouse infectivity of each virus was maintained for 20-132 days, and although original mouse brain suspensions were cumulatively diluted $10^{6-35}$ times, virus titers of cultural harvests remained $10^{-5.0}$ to $10^{-8.0}$ $LD_{50}$/ml for PK cultures and for weanling mice (Table I). Of

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† We are indebted to Swift Co. for weekly supply of porcine kidneys and to Hormel Fn. for kidneys from their specially bred line of miniature pigs.

TABLE I. Serial Passage of JE Virus in Porcine Kidney Cell Cultures (5 Experiments).

| JE virus strains | | | | | | Virus harvested from serial passages as determined by simultaneous titrations of | |
|---|---|---|---|---|---|---|---|
| Original source and designation | Mouse brain passage used as original inoculum | No. of virus passage in PK cultures | Duration of virus passage (days) | Total days in culture | Cumulative log dilution of mouse brain inoculum | CPE in PK cultures, $-\log TCD_{50}$/ml | Viral lethality in weanling mice, $-\log LD_{50}$/ml |
| Blood from pig in mosquito trap, Sagiyama, 21 Aug., '56 Pig-6 | 2 | [mouse brain inoculum] | | | | (6.3) | (8.9) |
| | | 1 | 4 | 4 | 4 | 4.5 | 7.3 |
| | | 6 | 4 | 19 | 11 | 6.6 | 6.0 |
| | | 11 | 3 | 35 | 16 | 6.3 | 5.1 |
| | | 18 | 4 | 61 | 25 | 7.0 | 5.2 |
| | | 21 | 3 | 69 | 28 | 5.5 | 4.7 |
| | | 24 | 4 | 83 | 31 | 5.2 | 6.3 |
| | | 27 | 4 | 93 | 34 | 4.8* | n.t.† |
| | | 28 | 4 | 97 | 35 | 5.8 | 5.0 |
| Blood from wild Black-crowned Night Heron, Shinhama, 29 July, '55 281801 | 4 | | | | | (7.5) | (9.3) |
| | | 6 | 4 | 23 | 7 | 6.7 | 5.0 |
| | | 7 | 4 | 27 | 8 | 5.0* | n.t. |
| Mosquito pool (14 *C. tritaeniorhynchus*), Sagiyama, 8 Aug., '55 M5/596 | 3 | | | | | (6.5) | (10.2) |
| | | 4 | 4 | 18 | 5 | 5.0* | n.t. |
| | | 5 | 2 | 20 | 6 | 6.5 | 5.4 |
| Human brain, Yokosuka, 5 Sept., '55 55-1735 | 3 | | | | | (5.5) | (9.2) |
| | | 6 | 2 | 20 | 7 | 6.6 | 5.8 |
| | | 8 | 4 | 27 | 9 | n.t. | 5.7* |
| Human spinal fluid, Japan, 1935 Nakayama | >40 (+41 egg passages) | 1 | 10 | 10 | 1 | 5.2 | 8.2 |
| | | 5 | 7 | 42 | 5 | 5.2 | 8.0* |
| | | 10 | 6 | 72 | 10 | 6.2 | 8.3 |
| | | 15 | 6 | 102 | 15 | 6.5 | 7.5 |
| | | 20 | 6 | 132 | 20 | 6.7 | 7.1 |

* CPE or mouse infectivity of virus was neutralized with specific JE virus rabbit antiserum.

† n.t. = not tested.

interest were changes in ratio, mouse $LD_{50}$/PK culture $TCD_{50}$, that occurred with serial passage of virus. Whereas each strain of virus as mouse brain suspension or first PK-passage harvest titrated significantly higher in weanling mice than in PK cultures, virus derived from fifth or higher passages in porcine kidney cells assayed approximately as high in PK cultures as in mice (Table I). This finding has significance since it indicates that PK-passaged JE virus can be detected as well in PK cultures as in weanling mice, and that PK-adapted virus could be employed for assay of serum neutralizing antibodies in PK cultures.

*Cytopathogenic effect of JE virus for porcine kidney cells and its neutralization by specific antibody.* Each of 4 strains of JE virus in low mouse passage (Table I) regularly destroyed 80-100% of PK cells in 2-5 days, even at terminal dilutions in virus titrations; Nakayama strain did not totally destroy cells until 6-10 days after inoculation. Cells first rounded, then aggregated in clusters, and finally detached from the glass. Usually peripheral cells of epithelial sheets were first affected, but central foci of degeneration were also seen. Uninoculated cells and cells undergoing destruction by JE virus are presented for comparison in Fig. 1 and 2.

The cytopathogenic effect as well as mouse infectivity of JE virus could be neutralized by specific rabbit antiserum. Each strain of virus employed for serial passage experiments

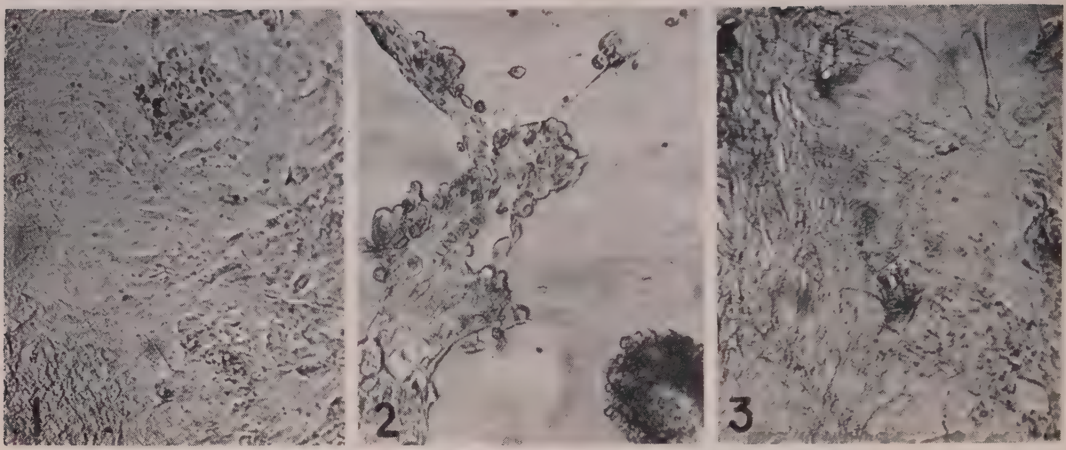

FIG. 1-3. Cytopathogenic effect of Japanese encephalitis virus (pig-6 strain of 27th passage, Table I) for porcine kidney cells in test tube cultures; neutralization of CPE by specific rabbit antiserum. Final magnification, × 100.

FIG. 1. Normal cells, uninoculated with virus.
FIG. 2. Cells 3 days after virus inoculation.
FIG. 3. Cells 3 days after inoculation of virus admixed with antiserum.

was neutralized either in PK cultures or in mice at passage levels indicated in Table I. Mouse infectivity of the human strain virus (55-1735) as 8th PK cultural harvest was reduced from $10^{4.2}$/0.03 ml without antiserum to $<10^{0.9}$/0.03 ml in the presence of antibody. A photograph of cells protected from destructive effects of pig-6 virus by antiserum is shown in Fig. 3.

*Growth curve of JE virus in porcine kidney cultures.* Growth curves were determined for 2 strains of JE virus in PK cultures to learn the optimal time for harvest of virus. Pig-6 and mosquito 5/596 strains of JE virus were inoculated as mouse brain suspensions into PK cultures in quantities calculated to yield 6.9 and 4.3 mouse $LD_{50}$ of virus/ml of cultural fluid immediately after inoculation. Quantities of mouse infectious virus in pooled cells and fluid were then determined 1, 3, 4, 5 and 7 days after inoculation. Virus grew in cultures of porcine kidney cells and reached maximal titers during 3-5 days after inoculation (Fig. 4). Thereafter essentially 100% of cells were destroyed and virus merely deteriorated.

*Discussion.* Until recently JE virus was in the group of arthropod-borne viruses which fail to exhibit a consistent cytopathogenic effect in tissue culture. Although some arbor viruses, such as Western equine encephalomyelitis and West Nile fever regularly destroy HeLa(7,8), Henle's human intestinal cells(8), monkey kidney(9), and chick embryonic tissue cultures(9), JE virus has with 2 exceptions failed to produce total and/or consistent CPE(7,8,10). The exceptions are the report of McCollum and Foley that 2 strains in 4th and 8th mouse passages incompletely destroy Detroit-6 human epithelial cells(10), and the recent discoveries of Kissling(11), and Diercks and Hammon(12) that high mouse passage Nakayama strain produces CPE and can be titrated in primary cultures of hamster kidney. The findings reported herein with porcine kidney cells thus provide a second cell culture system in which JE virus can be assayed. However, because porcine kidneys are available in almost unlimited supply and at little expense from abattoirs, they may prove to be under certain circumstances, potentially superior to hamster kidneys for mass production of virus and widespread, inexpensive utilization of tissue cultures in study of JE virus. Moreover, it is conceivable that PK cultures might isolate virus strains biologically different from the mouse pathogenic strains currently known, and that their inexpense and ease of mass production may make them equal or superior to mice in tests for neutralizing antibody. The possibility that a vaccine for Japanese encephalitis could be made either

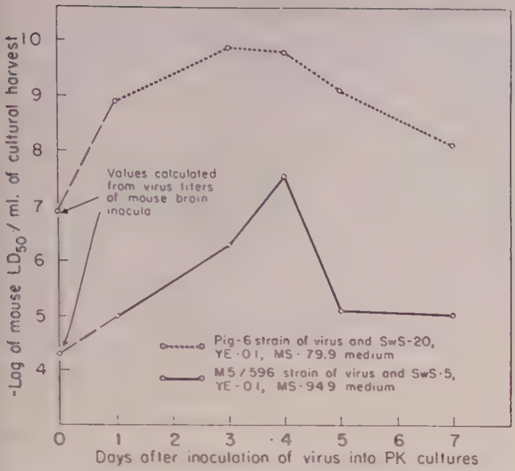

FIG. 4. Growth curves of Japanese encephalitis virus in porcine kidney cultures.

from inactivated or attenuated virus grown in porcine or hamster kidney cultures also deserves exploration because the mouse brain- and chick embryo-derived vaccines now available have been unsatisfactory for large scale use in humans. Since swine are plentiful in Asia, porcine kidneys would be readily available for vaccine production in countries where Japanese encephalitis occurs.

The observation that JE virus grows in porcine kidney cells *in vitro* cannot currently be related to the pathogenesis of swine infection. Although Japanese pigs are frequently infected in nature and develop viremia and antibodies (unpublished observations), they rarely manifest disease (encephalitis or stillbirth). Whether the kidney *in vivo* is a site of virus proliferation following natural infection by mosquito bite remains to be determined, although if virus actually grows in swine kidneys, it certainly does not destroy cells as extensively as *in vitro* since no obvious renal pathology occurs. Similar, incompletely understood, situations also exist with the viruses of Newcastle disease, fowl plague, hemagglutinating virus of Japan and vesicular exanthema which have recently been reported to grow in porcine kidney cultures(13-15).

*Summary.* Cells cultured *in vitro* from porcine kidneys support growth of JE virus and are concomitantly destroyed. Each of 5 virus strains (from pig, bird, mosquito and man) was propagated serially through 5-28 passages. Virus harvests from porcine kidney cultures titrated $10^{5-8}$ $ID_{50}$/ml in mice and in PK cultures. Total destruction of cells by recently isolated viruses in low mouse passage occurred usually within 2-5 days after inoculation of cultures, and could be prevented by specific antiserum.

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# Complement Levels in Mammals.* (24427)

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While investigating the level of complement in sera of patients with Hodgkin's disease and patients with carcinoma, the complement level in various experimental animals was determined so that these animal sera could be used to find complement-fixing antibodies. The level of serum complement for human beings and guinea pigs has been determined many times. Information about other species is limited, and determinations by a well-tested method such as that of Mayer and coworkers (1) are nonexistent. Since this method shows good precision and reproducibility(2), it has been employed with some simplifications for measuring mammalian complement, and it is useful whenever C′ levels determined in different laboratories are to be compared.

*Materials and methods.* (a) Sheep cells obtained commercially† were stored in Alsever's solution. They were washed, standardized, and sensitized as previously described(3) to give a 1.25% sensitized cell suspension in barbital-buffered saline with added Mg. (b) Hemolysin was obtained commercially† and titered as described(3). (c) Sera were obtained from blood allowed to clot for 2 hours at room temperature, rimmed, spun at 1200 g at 1°C for 10 minutes. Serum was removed and spun again under the same conditions for 30 minutes more to remove residual red cells. Complement titrations were carried out within 6 hours on this serum; simultaneously small portions were sealed in glass ampoules and stored in dry ice at minus 70°C. (d) A Coleman model 6 spectrophotometer was used. Cuvettes were Wasserman tubes, 16 x 100 mm, selected for uniformity and marked so that the same optical density was obtained with $CuSO_4$ solution as described (4). All operations were carried out in these tubes so that no transfers were made once all the reagents had been combined. These tubes gave the same results as 40 ml centrifuge tubes recommended previously(1,2) and were considerably more convenient. (e) All glassware was washed in trisodium phosphate solution, rinsed in tap water, distilled water, then allowed to remain overnight in distilled water, and dried. Duplicate analyses carried out both in tubes washed in dichromate-sulfuric acid and tubes washed in phosphate agreed within experimental error. (f) Complement titration was done as follows: 1) Human sera and sera from rabbit, hamster, rat and dog were diluted 1:100 with barbital-buffered saline; guinea pig serum 1:500; and at least three tubes set up for each sample. 2) Two milliliters of sensitized sheep cells were added to each tube. 3) Buffered saline was added to each tube in an amount calculated to give a final total volume of 7.5 ml when all reagents were present. 4) Usually 2.0, 2.5 or 3.0 ml of the serum dilution were added to the appropriate tube. 5) A blank of cells and saline only, as well as 2 duplicate tubes of cells plus water, corresponding to 100% hemolysis, were set up simultaneously. 6) Tubes were stoppered, shaken, incubated at 37°C for 40 minutes with frequent shaking, centrifuged at 1200 g for 10 minutes and optical density read at 550 mu. 7) Per cent hemolysis in each tube, milliliters of serum giving 50% hemolysis, and the units of C′ per milliliter were calculated as described previously(3). 8) Mouse serum was used undiluted and with one-fifth of the above quantities of cells and buffer in a total volume of 1.5 ml. Cuvettes used were 10 x 75 mm. Milliliters giving 50% hemolysis were multiplied by 5. Human sera assayed as described above gave the same values when repeated using this "micro" method.

*Results* for different species are shown in Table I. For mice, values were obtained graphically from the von Krogh equation(3) in its logarithmic form: $\text{Log } x = \log k + \frac{1}{n} \log \left( \frac{y}{1-y} \right)$ where x is milliliters of serum used, y is degree of hemolysis (100 y = %

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† Certified Blood Donor Service, Jamaica, N. Y.

TABLE I. Complement Levels in Mammals.

| Species | No. | $C'_{50}$ units/ml | Range |
|---|---|---|---|
| Normal humans | 65 | 39.2 ± 4.1* | 25.4 – 48.4 |
| Guinea pig | 11 | 262 ±36 | 218 –341 |
| Dog | 7 | 35.7 ± 7.9 | 21.7 – 47.3 |
| Hamster, Chinese *(Cricetelus griseus)* | 6 | 40.6 ± 7.7 | 32.8 – 55.1 |
| Rat (Long-Evans) | 10 | 32.4 ± 8.4 | 18.6 – 49.8 |
| Rabbit (New Zealand White) | 11 | 8.8 ± 1.7 | 6.5 – 11.6 |
| Mouse, C3H | 9 | .67 ± .38 | .39– 1.5 |

* Stand. dev.

hemolysis) and k and n are constants. When log x is plotted against log $\left(\frac{y}{1-y}\right)$ a straight line results, and its slope is $\frac{1}{n}$. C′ values for all other species were calculated using the von Krogh factors(3). All species except the C3H mice gave a slope of 0.2 ± 0.02 as indicated previously(1) for humans and guinea pigs. Normal C3H mice gave slopes of 0.38, 0.43, 0.59, 0.81 on four different occasions.

One human normal serum titered 8 times on the same day gave a value of 42.9 u/ml with a standard deviation of 1.1. Another one titered 5 times gave 40.2 ± 1.4. Storage at dry ice temperature did not affect C′ titers of normal or pathological sera for at least 2 months; by 6 to 9 months titers had decreased about 15%. Titers on freshly drawn, unfrozen serum were the same as on those portions frozen immediately after shedding and assayed the next day. Complement titers in 10 normal humans determined at different times on freshly drawn samples during the course of a year were constant within 10%.

Of interest is the fact that the mouse, previously reported(5) to be void of or very low in C′, does have a readily measurable amount. Furthermore, the same hemolytic system used to assay human C′ can be used on the mouse without any modifications, contrary to the report of McGhee(5), who had to modify the system in order to find measurable amounts of C′ in Swiss albino mice. Since it has been shown(1) that hemolytic titers will vary greatly with the same number of sheep cells used, the system volume, and ionic concentrations, it is important when making comparisons that the same system be used throughout. We have also used this hemolytic system for measuring the fixing of guinea pig complement in the presence of antibody-antigen reactions.

Relative C′ levels found by Reader(6) were in the ratio of 1:1:4 for humans, rats and guinea pig, compared to 1:0.8:7 in this study. Larin *et al.*(7) found a ratio of 1:4 for dogs and guinea pigs compared to 1:7 found here. Values for humans and guinea pigs in this work agree well with those reported by investigators using the same technic(1,2).

*Summary.* 1. A sensitive, reproducible method capable of measuring complement levels in 7 different mammalian species is described. This method is a modification of the technic of Mayer and co-workers(1). 2. In sera of hamster, rat and dog the hemolytic complement activity is about the same as in that of normal humans; in guinea pig serum it is 7 times greater; in rabbit serum it is one-fourth of that in humans and in mice it is one-sixtieth, although readily measurable. 3. The slope of the hemolytic curve for C3H mice differs from that of all other species examined and also varies for individual mice.

I am indebted to Dr. Robert Kassel, of this laboratory, who provided various animal bloods, and to A. Bianco and A. M. Ehnes for technical assistance.

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# Influence of Sex and Dietary Phosphatides on Cholesterol Levels in Blood Plasma of Swine.* (24428)

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Substitution of vegetable oils for more saturated fats in the diet has been reported to reduce blood cholesterol in various animals and in man(1-5). A temporary reduction of serum cholesterol in human subjects was observed by Steiner and Domanski(6) following ingestion of a commercial soybean lecithin mixture. Pilgeram and Greenberg(7) observed increased phosphatidyl choline formation in rat liver slices from labeled aminoethanol and methionine, when rats were fed high cholesterol diets, in contrast to rabbits, a species more susceptible to atherosclerosis, suggesting that phosphatidyl choline is necessary for normal cholesterol metabolism. These authors(8) also suggested that inability of certain animal species to form lecithin may result in metabolic block producing atherosclerosis and that certain phosphatides may be necessary for normal cholesterol breakdown. More recently it has been reported that injection of lecithin into rabbits grown on atherogenic diets results in clearing of atherosclerotic lesions(9). We studied the effects of incorporating certain soybean phosphatide fractions in the diet on plasma cholesterol levels of a newly developed breed of small swine (10).

*Materials and methods.* Thirty miniature pigs were divided into 5 groups of 6 pigs each when pigs were 40 to 51 days old. Group 1 consisted of 4 boars and 2 gilts. Each other group consisted of 3 boars and 3 gilts. The boars were not castrated. It was of interest to observe sex differences, if any, in blood cholesterol values, as well as possible sex differences in response to treatment. The diets are shown in Table I. The pre-test diet was fed 33 days. Then, for the next 6 weeks, group 1 was fed the low-fat diet (5.17%) and the other 4 groups the high-fat diet (18.17%). At this time soybean phosphatides of 3 types were added at the level of 2% to diets of 3 groups fed the high-fat diet. Compositions of phosphatides were estimated to be as follows:‡ (a) Crude soybean phosphatide, estimated to approximate ⅓ phosphatidyl choline, ⅓ phosphatidyl ethanolamine, and ⅓ inositol phosphatide. Methyl esters of phosphatide fatty acids had an iodine value of 126.6, and contained 43.6% dienoic acids and 6.4% trienoic acids, measured by alkali isomerization-spectrophotometric technic(11). (b) Alcohol-insoluble fraction of soybean phosphatide, estimated to approximate ⅔ inositol phosphatide, ⅓ phosphatidyl ethanolamine, and trace of phosphatidyl choline. Methyl esters of the phosphatide fatty acids had an iodine value of 121.1, and contained 41.1% dienoic acids and 5.3% trienoic acids. (c) Alcohol-soluble fraction of soybean phosphatide, estimated to approximate ⅔ phosphatidyl choline, ⅓ phosphatidyl ethanolamine, and a trace of inositol phosphatide. The methyl esters of phosphatide fatty acids had iodine value of 104, and contained 21.1% dienoic acids and 2.7% trienoic acids. Phosphatide supplements were fed 17 weeks. All diets were fed isocalorically based on total body weight of all pigs, weighed weekly. The experiment was started with 270 calories/kg body weight/day, but because of continuous decrease in daily caloric consumption/kg body weight, it was necessary to reduce this level steadily as pigs grew older. At end of 17-week test period, the pigs were consuming 100 calories/kg body weight/day. Plasma cholesterol levels were determined(12) at 3 week intervals. Only data from end of pre-test period, the end of basal period, and end of test period are given in Table II. Data obtained at intermediate in-

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‡ Phosphatide fractions and estimates of their composition were furnished by The Glidden Co. through the courtesy of H. T. Iveson.

TABLE I. Composition of Rations.

| Ingredient | Diet<br>Pre-test | <br>Low-fat | <br>High-fat |
|---|---|---|---|
| | % | | |
| Corn, ground | 15 | 15 | 15 |
| Oat groats | 30 | 30 | 30 |
| Soybean oil meal, solvent | 20 | 20 | 20 |
| Dried skim milk | 10 | 10 | 10 |
| " fish solubles | 2 | 2 | 2 |
| Trace mineralized salt | .5 | .5 | .5 |
| Ca-P mineral mix (28% Ca, 6% P) | 1 | 1 | 1 |
| Steamed bone meal | 1 | 1 | 1 |
| Fortafeed 2-49c | .2 | .2 | .2 |
| Vit. A and D oil (2250 A, 400 D) | .3 | .3 | .3 |
| Purified beef tallow | .0 | 2 | 15 |
| Cellulose (Alphacel) | .0 | 2.5 | 2.5 |
| Cholesterol | .0 | .5 | .5 |
| Cerelose (dextrose) | 20 | 15 | 2 |
| Calculated analysis: | | | |
| Calories/kg | 3419 | 3099 | 3746 |
| Protein, % | 19.12 | 19.12 | 19.12 |
| Fat, % | 3.17 | 5.17 | 18.17 |
| Calcium, % | .72 | .72 | .72 |
| Phosphorus, % | .61 | .61 | .61 |

tervals were consistent with the data shown in the Table. Analysis of variance was made for plasma cholesterol values and body weight values for each bleeding period. Plasma lipides were extracted by cold saponification, 1 N KOH, in ethanol. Six to 8 volumes of pooled plasma were mixed with 20 volumes of alcoholic KOH and left at room temperature over night. The mixture was then brought to boiling temperaure for 15 min. and, after cooling, the fatty acids were isolated and taken to final volume of 10 ml with absolute methanol. All operations were carried out under a blanket of nitrogen. Polyunsaturated fatty acids were analyzed according to a modification of the method of Holman *et al.*(11). The final calculations were based on constants of Herb and Riemenschneider(14). Plasma phospholipide phosphorus analysis was performed by modification of the method of King(13). Phospholipides were estimated as 25 times the concentration of lipide phosphorus. In each group equal volumes of plasma, from males or females, were pooled for analyses of polyunsaturated fatty acids.

*Results.* Differences in body weights were nonsignificant for all periods tested, suggesting that neither differences in dietary treatment nor in sex of animals had any effect on changes in body weight.

Plasma cholesterol values of pigs in all groups increased similarly during the 6 weeks prior to incorporation of phosphatide supplements, the males averaging +50 mg% and the females +58 mg%.

Plasma cholesterol values throughout test period showed significant variation in relation to sex, with females showing higher plasma cholesterol values than males, regardless of dietary treatment. By end of third week the significance reached the 5% level, and by the

TABLE II. Blood Plasma Cholesterol.

| Group | | At end of<br>33-day pre-test period | <br>6-wk basal period | <br>17-wk test period | Change during 17-wk test period |
|---|---|---|---|---|---|
| | | mg % | | | |
| Low fat | ♂ (4) | 67 | 109 | 80 | −29 |
| | ♀ (2) | 97 | 144 | 123 | −21 |
| | Avg | 77 | 121 | 94 | −27 |
| High fat | ♂ (3) | 78 | 110 | 119 | +9 |
| | ♀ (3) | 100 | 140 | 166 | +26 |
| | Avg | 89 | 125 | 142 | +17 |
| *Idem* + 2% crude lecithin | ♂ (3) | 83 | 146 | 84 | −62 |
| | ♀ (3) | 91 | 176 | 186 | +10 |
| | Avg | 87 | 161 | 135 | −26 |
| " + 2% alcohol-insoluble | ♂ (3) | 102 | 160 | 121 | −39 |
| | ♀ (3) | 78 | 148 | 142 | −6 |
| | Avg | 90 | 154 | 132 | −22 |
| " + 2% alcohol-soluble | ♂ (3) | 99 | 154 | 109 | −45 |
| | ♀ (3) | 93 | 139 | 174 | +35 |
| | Avg | 96 | 147 | 141 | −6 |

TABLE III. Plasma Polyenoic Acids at End of Basal Period.*

| Group | | Dienes | Trienes | Tetra-enes | Penta-enes | Hexa-enes | PL† | PL/TC‡ |
|---|---|---|---|---|---|---|---|---|
| | | mg % | | | | | | |
| Low fat | ♂ | 23.5 | .7 | 11.4 | 3.2 | 2.3 | 138.5 | 1.27 |
| | ♀ | 15.5 | 1.3 | 11.1 | 2.5 | 2.9 | 139.0 | .97 |
| High fat | ♂ | 18.6 | .6 | 8.2 | 2.9 | 2.2 | 173.0 | 1.57 |
| | ♀ | 24.6 | .6 | 13.7 | 3.6 | 3.2 | 176.0 | 1.26 |
| *Idem* + 2% crude lecithin | ♂ | 14.3 | .0 | 6.7 | 2.3 | 1.7 | 168.4 | 1.15 |
| | ♀ | 21.3 | 1.0 | 12.6 | 3.6 | 3.7 | 201.7 | 1.14 |
| " + 2% alcohol-insoluble | ♂ | 16.8 | .0 | 11.6 | 3.6 | 3.9 | 191 | 1.19 |
| | ♀ | 15.6 | .0 | 13.9 | 3.6 | 4.7 | 166 | 1.12 |
| " + 2% alcohol-soluble | ♂ | 28.9 | .1 | 15.1 | 4.2 | 4.9 | 158 | 1.03 |
| | ♀ | 16.9 | .3 | 12.4 | 3.5 | 3.7 | 152 | 1.09 |

* Pooled plasma polyenoic acid content, mg/100 ml, at end of 6-wk basal period.
† Phospholipides—mg/100 ml plasma.
‡ Phospholipide (PL)/total cholesterol (TC) ratios.

end of sixth week, the 1% level. This sexual difference in plasma cholesterol probably was due to the fact that all females were in gestation by end of experiment. In this regard it should be noted that pregnant dairy cattle have been reported to have higher cholesterol values than nonpregnant cattle(15).

Although plasma cholesterol values of all females combined were significantly higher than for males, the mean values actually were lower in 2 groups, the low-fat basal Group 1, and the high-fat basal group receiving 2% alcohol-insoluble supplement (Group 4). Statistical significance of differences in plasma cholesterol values due to dietary treatment was low ($P>.05$, $P<.10$). However, data in Table II showed definite trends, all of which were consistent except for the 3 females in Group 4. During the test period, plasma cholesterol values of both males and females decreased in the low-fat Group 1 and increased in the high-fat Group 2. That phosphatide supplements had an effect on plasma cholesterol is shown since in all 3 supplemented groups, values for males decreased appreciably. Even though, overall, females showed some increase in plasma cholesterol, those in Group 4 (supplement containing 66% inositol phosphatide) actually decreased slightly. The fact that the supplement containing large amount of inositol phosphatide led to a marked decrease in plasma cholesterol in males and inhibited increases in plasma cholesterol of females, suggests that this supplement was the most active in reducing plasma cholesterol.

During test period, plasma tetraenoic acids (arachidonic) fell in all groups, and dienoic acids (linoleic) increased in both males and females fed high fat diets (Tables III and IV). Females fed basal diets had plasma cholesterol levels equal to, or greater than, males maintained on same diets. During the test period, females maintained higher plasma tetraenoic acid levels than males. There was no apparent correlation between plasma cholesterol levels and circulating polyunsaturated fatty acids. While plasma phospholipides fell in males fed phosphatide supplements, such changes were not apparent in females. Pregnant females did not show any change in ratio of their plasma phospholipide to cholesterol. In contrast, males receiving phosphatide supplements showed a marked decrease in plasma phospholipides and in their phospholipide-cholesterol ratios. Longer term studies are in progress to determine more specifically the changes in tissue lipides of miniature pigs fed phosphatides.

*Summary.* 1) Body weights and blood plasma cholesterol values were observed periodically in male and female miniature swine fed high-tallow diets supplemented with dietary soybean phosphatides, for 17 weeks. Body weight changes were uniform throughout and were not related to sex or dietary treatment. The plasma cholesterol values of females as a whole after 3 weeks on the test, were significantly higher than for males ($P<.05$) and remained so after 6 weeks on test diets (P

TABLE IV. Plasma Polyenoic Acids at End of Test Period.*

| Group | | Dienes | Trienes | Tetra-enes | Penta-enes | Hexa-enes | PL† | PL/TC‡ |
|---|---|---|---|---|---|---|---|---|
| | | mg % | | | | | | |
| Low fat | ♂ | 17.7 | .0 | 2.4 | 1.3 | .1 | 121.5 | 1.52 |
| | ♀ | 19.1 | .1 | 6.9 | 2.9 | 3.7 | 195.6 | 1.59 |
| High fat | ♂ | 34.6 | .8 | 3.3 | 1.8 | .1 | 170.8 | 1.49 |
| | ♀ | 27.2 | .7 | 6.1 | 1.8 | 2.3 | 185.8 | 1.12 |
| *Idem* + 2% crude lecithin | ♂ | 32.3 | .5 | 2.5 | .1 | .1 | 109.2 | 1.30 |
| | ♀ | 79.6 | .0 | 15.8 | 3.9 | 6.7 | 192.0 | 1.03 |
| " + 2% alcohol-insoluble | ♂ | 60.2 | 1.1 | 6.6 | 2.8 | 2.4 | 100.5 | .85 |
| | ♀ | 32.9 | .6 | 9.4 | 2.7 | 3.8 | 150.8 | 1.06 |
| " + 2% alcohol-soluble | ♂ | 47.5 | .9 | 8.1 | 2.9 | 2.5 | 140.2 | .78 |
| | ♀ | 23.6 | .0 | 9.4 | 3.3 | 5.3 | 181.8 | 1.05 |

* Pooled plasma polyenoic acid content, mg/100 ml, at end of 17-wk test period.
† Plasma phospholipides, mg/100 ml.
‡ Phospholipide (PL)/total cholesterol (TC) ratios.

<.01), when gilts were pregnant. 2) While differences in plasma cholesterol values of several groups, due to dietary treatment, were not statistically significant at the 5% level, definite trends were observed. Plasma cholesterol values of all pigs fed the low-fat diet were reduced during test period, while those fed the high-tallow diet increased slightly. All 3 of the dietary soybean phosphatide supplements, when added to the high-tallow diets, reduced plasma cholesterol values of males. The alcohol-insoluble fraction supplement (⅔ inositol phosphatides) also slightly reduced plasma cholesterol values of females. 3) Thus, under conditions employed, soybean phosphatides incorporated in the diet appeared to counteract the hypercholesteremic effect of the diet containing a relatively high proportion of a saturated fat. 4) The tetraenoic acid concentration of plasma tended to be higher in females than in males fed comparable diets. The feeding of phosphatide supplements, while having little effect on plasma lipides of pregnant miniature pigs, promoted a marked drop in plasma cholesterol, phospholipides, and phospholipide-cholesterol ratios in male miniature pigs. There was no apparent correlation between changes in plasma polyunsaturated acids and changes in other plasma lipides during this study.

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# Protective Effect of Seaweed Extracts for Chicken Embryos Infected with Influenza B or Mumps Virus. (24429)

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In screening tests for antiviral activity we often observed that insoluble compounds prepared as a suspension in 0.25% agar exhibited erratic, minimal protection for chicken embryos infected with influenza B virus. We suspected the presence of an active material in the agar, the more so, because different batches gave different results. This prompted the present study of the polysaccharides obtained from marine algae, particularly *Gelidium cartilagenium,* the common source of domestic agar, and carrageenan, a galactan derived from *Chondrus crispus.* Polysaccharides from various sources exhibit certain antiviral properties. Bacterial polysaccharides have shown marked antiphage activity (1,2) and a yeast polysaccharide inhibited the multiplication of tobacco mosaic virus(3). The modifying effect of bacterial polysaccharides on infections caused by animal viruses was first observed by Horsfall and McCarty(4) and later by Ginsberg, *et al.*(5). They demonstrated the marked antiviral specificity of these substances. Similarly apple pectin had specific effects against influenza A virus in fertile eggs(6).

*Materials and methods. Seaweed extracts. Gelidium cartilagenium*: Agar obtained from this and other species of seaweed is mainly a linear polysaccharide of D-galactose units linked 1→3, with a small number of ethereal sulfate groupings(7). A typical extract was prepared as follows: 56 g of desiccated seaweed* was allowed to swell in 4 liters of distilled water, homogenized for 1 hour in Waring blendor, and shaken for at least 24 hours in a stoppered bottle. The insoluble materials were separated by centrifugation and filtration through paper, after which they were held for future extraction. After the filtrate had been concentrated *in vacuo* (in a falling film evaporator at 40°C) to 1 liter, the polysaccharide was precipitated by addition of 2.5 l of acetone. The collected precipitate was redissolved by warming in 125 ml of distilled water and the solution lyophilized to yield 3.75 g of a colorless solid. The insoluble seaweed material, processed a second and third time, yielded 1.86 g and 0.75 g of lyophilized solid, respectively. *Chondrus crispus*: Aqueous extracts of this alga yield the commercial product, carrageenin, which when purified is called carrageenan. It is a polysaccharide consisting principally of D-galactose units with some L-galactose and a ketose present. The amount of ester sulfate is about 28% greater in carrageenan than in agar. Carrageenin† was dissolved in water and acetone precipitated as described under *Gelidium* above. The lyophilized samples prepared from both sources gave typical test reactions for polysaccharides and were low in ash (2 to 5%). The component associated with antiviral activity was non-dialyzable. *Solutions.* Antibiotic broth was prepared by addition of 100 units of penicillin and 100 mcg of dihydrostreptomycin/ml of tryptose phosphate broth. Glucosol solution was prepared as described by Fulton and Armitage(8). *Viral strains.* The WS and PR8 strains of Influenza type A, Lee strain of Influenza type B, Lasota strain of Newcastle disease virus (NDV) and Enders strain of mumps virus were employed. All except mumps virus cause lethal infections in 10-day-old embryonated chicken eggs. These viral strains were stored as infected allantoic fluid in sealed ampules a –70°C. *Titrations of infectivity.* Infected allantoic fluids were diluted in 10-fold steps in antibiotic broth. Groups of four 10-day-old embryonated eggs were inoculated intra-allantoically with 0.2 ml of each dilution and incubated at 36°C for 2 days or, in the case of mumps, for 6 days. The presence of viral hemagglutinins in the

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* Supplied through courtesy of American Agar and Chemical Co., San Diego, Calif.

† Seakem No. 6, obtained through courtesy of Seaplant Chemical Corp., New Bedford, Mass.

TABLE I. Effect of Algal Polysaccharides on Viral Infections in Chicken Embryos (Treatment and Infection by the Allantoic Route).

| Material inj. 1 hr after infection | Mumps | Influenza | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Lee | | PR8 | | WS | | NDV | |
| | I/T* | S/T† | AST‡ | S/T | AST | S/T | AST | S/T | AST |
| 1.25 mg carrageenan extract | 2/10 | 9/10 | >10.0 | 0/10 | 5.6 | 0/10 | 4.4 | 1/10 | 6.7 |
| 1.25 mg *Gelidium* extract | 3/10 | 7/10 | 9.6 | 0/10 | 5.8 | 0/10 | 4.6 | 0/10 | 6.9 |
| .5 ml antibiotic broth | 10/10 | 0/10 | 4.2 | 0/10 | 5.2 | 0/10 | 4.7 | 0/10 | 6.7 |

* I/T = No. of infected/total; 100 $EID_{50}$.
† No. of survivors/total; 100 $LD_{50}$.
‡ Avg survival time in days.

allantoic fluids indicated infection, and the $EID_{50}$ (50% egg infectious dose) was calculated by the method of Reed and Muench(9). The hemagglutination test was done by the spot plate method(10) or according to the method of Salk(11) when quantitative data were desired. *Embryonated egg tests.* Groups of 8-day-old embryonated chicken eggs were infected by the allantoic route with approximately 100 $EID_{50}$ of mumps virus diluted in antibiotic broth. After an incubationary period of 6 days at 36°C the number of infected embryos was determined by the presence of hemagglutinins in the allantoic fluids. Influenza B virus, Influenza A virus or NDV diluted in antibiotic broth to 100 $LD_{50}$ (50% lethal dose) was injected into the allantoic sac of 10-day-old chicken embryos. The eggs were incubated at 36°C for 10 days and embryonic deaths were recorded daily. Each test and control group consisted of 8 to 14 fertile eggs. One hour after infection with either virus, or as indicated in the text, 0.5 ml of the test material was inoculated by the allantoic route. The control groups received 0.5 ml of antibiotic broth. *De-embryonation technic.* The combined *in ovo-de-embryonation* technic as described by Finter, *et al.*(12) was employed.

*Results. A. Effect of algal polysaccharides on viral infections in chicken embryos.* The algal polysaccharides at a dose of 1.25 mg/embryo suppressed multiplication of mumps virus in 70-80% of the infected embryos and protected 70-90% of the embryos against death from influenza B virus infections (Table I). On the other hand, no protection was demonstrable under identical experimental conditions against infections with NDV and 2 strains of influenza A virus. Similar results were obtained when treatment preceded the infection by 1 hour or 24 hours. None of the polysaccharide-treated uninfected control embryos died and all hatched normally. Furthermore, histological examination of the chorioallantoic membranes showed no microscopic changes as a result of treatment by either polysaccharide.

*Dose response.* To determine minimum effective dose of polysaccharide, varying amounts of both polysaccharides were injected at doses ranging from 5 to 1280 mcg/fertile egg infected with influenza B virus. As shown in Fig. 1, 40 mcg carrageenan extract/embryo gave almost maximum protection (about 80%), whereas 5 mcg protected about 30% of the embryos. The results obtained with preparations of *Gelidium cartilagenium* were similar in the region of higher dose levels but little or no activity was found when less than 80 mcg was employed. Using 10-fold dilution of influenza B virus, the results, summarized in Table II, show that 1000 times

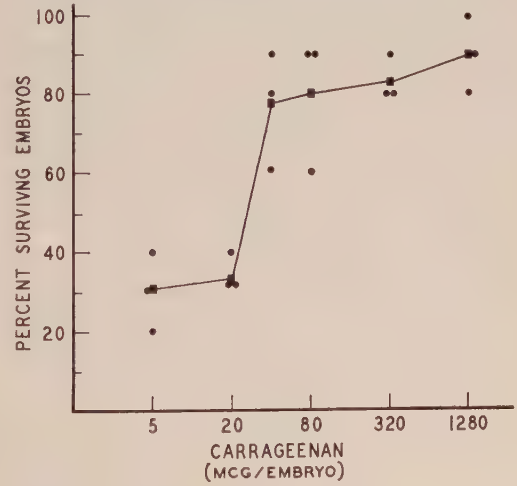

FIG. 1. Effect of varying amounts of carrageenan extract on chicken embryos infected with 100 $LD_{50}$ of influenza B virus. Curve represents arithmetic mean of 3 experiments.

TABLE II. Infectivity Titration of Influenza B Virus in Chicken Embryos Treated with Carrageenan Extract (Treatment and Infection by the Allantoic Route).

| Virus dilutions | Control embryos,* S/T | Treated embryos,† S/T |
|---|---|---|
| $10^{-4}$ | 0/10 | 1/10 |
| $10^{-5}$ | ” | 5/10 |
| $10^{-6}$ | ” | 9/10 |
| $10^{-7}$ | ” | 10/10 |
| $10^{-8}$ | 5/10 | ” |
| $10^{-9}$ | 10/10 | ” |
| 50% virus titration end point | $10^{-8.0}$ | $10^{-5.0}$ |

* 0.5 ml antibiotic broth 1 hr after infection.
† 78 $\mu$g carrageenan extract 1 hr after infection.

more virus was required to cause a 50% fatal infection in embryos treated with 78 mcg carrageenan extract than in controls.

*Effect of delayed treatment*: Groups of 14 randomly selected embryonated eggs infected with influenza B virus were treated at 1, 18 or 24 hours after infection with 40 mcg/egg of carrageenan extract. Four eggs from each group were used for repeated withdrawal of 0.2 ml allantoic fluid at various intervals up to 72 hours, and the amount of virus in these fluids was determined by egg infectivity titration (Fig. 2). Multiplication of virus in the 14 controls began after a lag period of 6 hours and viral concentration reached a peak titer of $10^{9.3}$ at 48 hours. On the other hand, treatment with polysaccharide caused a temporary suppression in viral multiplication for 48 hours; this was most marked when treatment had been started 1 hour after infection. All embryos of the control group died between 4 and 5 days after infection, whereas 40 to 80% of treated embryos survived. Similar results were obtained in experiments with extracts of *Gelidium cartilagenium*. In an identical experiment using the PR8 strain of influenza A virus, no significant differences in viral titers were noted in the treated and control groups, and none of the embryos survived the 10-day period of observation.

*Effect of treatment through the yolk sac*. Groups of 7-day-old chicken embryos were injected into the yolk sac with 1.25 mg algal polysaccharides. After 1 hour or after 3 days 100 $LD_{50}$ of influenza B virus was injected into the allantoic sac. No antiviral effect was demonstrable in two such experiments.

*B. In vitro effects. Effects on viral infectivity*: For this study the carrageenan or *Gelidium* extracts were dissolved at a dose of 80 mcg in 4.5 ml of allantoic fluid containing 100 $LD_{50}$/ml of influenza B virus, the mixtures were incubated at 36°C, and aliquots were removed for viral infectivity titration at 0, 1 and 4 hours. Since each test material showed the same viral concentration as the control specimens without polysaccharide, we concluded that these polysaccharides exerted no virustatic or virucidal effect *in vitro*.

*Effects on hemagglutination*. Green and Woolley(6) suggested that with influenza A a correlation exists between the inhibition of multiplication and of hemagglutination by polysaccharides. To examine this problem the effects of these algal polysaccharides were studied both on the chicken red blood cell as well as on the viruses (NDV, mumps, and the WS, PR8 and strains of influenza) themselves. 0.3 ml of packed chicken red blood cells was added to 2 ml of saline containing 1280 mcg of polysaccharides. The mixture was incubated at room temperature for 3 hours with frequent agitation. The cell suspension was centrifuged, washed 3 times with saline, and then resuspended in saline to 0.5% concentration. Agglutination did not occur at any

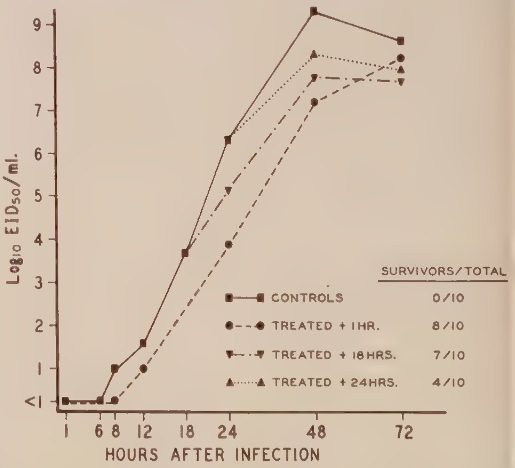

FIG. 2. Virus content and survival rates of chicken embryos treated with 40 $\mu$g of carrageenan at various intervals following infection with 100 $LD_{50}$ of influenza B virus. (Each point represents geometric mean of viral titers of 4 individual allantoic fluid samples.)

time in the absence of virus, but these polysaccharide-treated cells were agglutinated by 4 agglutinating units of each virus. Two-fold serial dilutions ranging from 1280 to 10 mcg of each extract/0.25 ml of saline were prepared and added to an equal volume of 4 agglutinating units of each virus in saline. The mixtures were held at room temperature for one hour before adding 0.5 ml of a 0.5% suspension of chicken red blood cells. No inhibition of hemagglutination was noted at any concentration of the polysaccharides.

Horsfall and McCarty(4) reported a modifying effect of intranasally administered agar upon intranasal infections with pneumonia virus of mice (PVM). A limited study was therefore performed with these algal polysaccharides in 3- to 4-week-old albino Swiss mice which had been infected intranasally with 100 $LD_{50's}$ of PVM or of influenza viruses (PR8 and Lee strain). Intranasal instillations of 60 mcg of polysaccharide per mouse, given 24 hours before or up to 4 hours after infection with lethal doses of PVM, protected 70% of the animals, but had no effect upon the course of infections with influenza A or B. Furthermore, no modifying effect was observed with either polysaccharide given intravenously or intraperitoneally.

*Discussion.* The antiviral effects of polysaccharide-containing substances extracted from *Gelidium cartilagenium* or carrageenan on chicken embryos were rather specific. These agents were non-toxic in the concentrations employed, and caused a transient suppression of growth of influenza B virus and a reduction of the infectivity of mumps virus; they were, however, without effect upon influenza A virus or NDV. The results obtained suggest the possibility that these polysaccharides may act intracellularly.

Ginsberg(5) postulated that PVM and mumps viruses require metabolic systems different from NDV and influenza virus, because polysaccharide derived from *Klebsiella pneumoniae* selectively inhibited only the former pair. Since the results of this study have demonstrated that extracts of *Gelidium cartilagenium* and carrageenan selectively interfere with the multiplication of PVM, mumps and influenza B virus, it would appear that these viruses may share metabolic pathways which can be blocked by certain polysaccharides.

Other algal polysaccharides, such as laminarin, fucoidin and algin, which lack galactan units in their chemical composition, showed no antiviral properties(6,13).

*Summary.* 1) Polysaccharides extracted from *Gelidium cartilagenium* and carrageenin exerted a marked inhibitory effect on growth of influenza B virus and mumps virus in embryonated eggs but had no effect on the multiplication of influenza A virus and NDV. Significant protection against otherwise fatal infections with influenza B virus was obtained when treatment was delayed as long as 24 hours after viral inoculation. 2) Use of commercial agar as a vehicle for insoluble compounds may yield misleading results in screening tests in chicken embryos infected with either influenza B or mumps virus. The use of inert agents such as carboxymethyl cellulose obviates this difficulty.

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## Steroid $\Delta^4$-Hydrogenases of Rat Liver.* (24430)

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Previous studies with fractions prepared from livers of adult male rats have demonstrated presence of both a soluble $\Delta^4$-$5\beta$-hydrogenase and a microsomal $\Delta^4$-$5\alpha$-hydrogenase(1). Development of a quantitative method for assay of these enzymes(2,3) has made possible a study of the effective enzyme concentrations in liver with respect to such factors as sex, age and endocrine status.

*Methods.* Animals were sacrificed by stunning and decapitation and the livers rapidly removed and chilled on ice. All subsequent operations were carried out at 2-3°C. Tissues were homogenized by a VirTis blendor at full speed for 20 seconds with one or 1.5 volumes of either 0.25 M sucrose or 0.154 M KCl as homogenizing medium. The various tissue fractions were prepared by differential centrifugation in a Spinco Model L centrifuge. The usual incubation procedure was as follows: a volume of each tissue preparation equivalent to 100 mg wet weight of whole liver plus 0.5 $\mu$M of steroid substrate was incubated with 1 $\mu$M TPN, 5 $\mu$M glucose-6-$PO_4$, 0.5 K unit glucose-6-$PO_4$ dehydrogenase, 10 $\mu$M $MgCl_2$, 20 $\mu$M phosphate buffer pH 7.2, and 0.154 M KCl to make a final volume of 1 ml; the incubation periods were for 10 minutes (unless otherwise indicated) at 37° in an atmosphere of nitrogen. The reaction was stopped by rapid chilling of the incubation tubes and steroid substances were extracted with 4 ml redistilled methylene dichloride. Recoveries in control experiments averaged 95%. This value was used in calculation of reaction rates which were determined by measuring the loss of the characteristic absorption near 240 m$\mu$. All determinations were performed in triplicate. Nitrogen determinations were made in duplicate by a micro-Kjeldahl method.

*Results. Comparison of reducing activity of male and female rat livers.* A marked quantitative difference was observed in steroid $\Delta^4$ reducing activity of whole homogenates prepared from livers of adult male and female rats when 11-deoxycortisol was used as a substrate. The activity of female livers was consistently higher whether expressed as $\mu$M of substrate reduced /100 mg wet weight equivalent or /mg nitrogen (Table I). In addition to the quantitative difference, a striking qualitative difference was observed. In the female, $\Delta^4$ reducing activity was found only in the microsomal fraction. No evidence for the $5\beta$ soluble enzyme could be obtained in female liver preparations but this enzyme is easily demonstrable in male rat liver(1,4). The difference in level of $\Delta^4$ reducing activity observed in whole homogenates of male and female livers was also seen when the activity of the microsomal fractions was compared (Table I). Ratio of activity (female/male) was only slightly lower than that seen with whole homogenates.

The microsomal $5\alpha$-enzyme prepared from the female liver appeared to be the same as that separated from the male liver with respect to pH optimum (7.0-7.5) stability and similar relative rates of reduction with various steroid substrates (Table II). It may be noted that the ratio of $\Delta^4$ reducing activity between male and female was quite constant, whether the activities were compared in whole homogenates (not shown) or in microsomal fractions. This suggests that under the conditions of these experiments $\Delta^4$ reducing activity of male rat liver is predominantly due to the $5\alpha$ microsomal enzyme. Indeed, when the soluble enzyme was prepared free from particulate material by centrifugation at 105,000 x g, only 18-21% of the whole homogenate activity was found in this fraction. In confirmation of the findings of Tomkins(4), the soluble $5\beta$ enzyme was found to exhibit an acid pH optimum (5.5-6.0) and in the un-

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TABLE I. Comparison of Reducing Activity of Whole Homogenates and Microsomal Fractions of Male and Female Rat Livers. Substrate: 11-Deoxycortisol.

| Type of preparation | No. of pairs | $\mu M \times 10^3$ steroid reduced/100 mg wet wt equivalent (mean ± S.E.) | | | $\mu M \times 10^3$ steroid reduced/mg nitrogen (mean ± S.E.) | | |
|---|---|---|---|---|---|---|---|
| | | ♂ | ♀ | Ratio* | ♂ | ♀ | Ratio* |
| Whole homogenate | 6 | 94 ± 8.3 | 349 ± 30.0 | 3.8 ± .44 | 76 ± 6.5 | 274 ± 27 | 3.7 ± .31 |
| Microsomal | 6 | 53 ± 5.4 | 188 ± 33.3 | 3.5 ± .44 | 147 ± 14.7 | 536 ± 137 | 3.4 ± .60 |

* Ratio = female/male.

TABLE II. Relative Rates of $\Delta^4$-Reduction ($\mu M$ per Min.) of Various Natural and Synthetic Steroids by Microsomal $5\alpha$ Enzyme Prepared from Male and Female Rat Livers and by Soluble $5\beta$ Enzyme from Male Rat Liver. (Results expressed in terms of cortisol = 100. Incubations were for 30 min. with 80 $\mu g$ steroid. Actual reductions of cortisol were 0.105, 0.112 and 0.097 $\mu M$ in 30 min. for female $5\alpha$, male $5\alpha$ and male $5\beta$ enzyme respectively.)

| Substrate | Type and source of enzyme preparation | | |
|---|---|---|---|
| | ♀ | ♂ | |
| | microsomal | Microsomal | Soluble |
| Cortisol | 100 | 100 | 100 |
| 11-Deoxycortisol | 91 | 80 | 67 |
| Deoxycorticosterone | 154 | 141 | 34 |
| Progesterone | 126 | 130 | 35 |
| $\Delta^1$-Dehydrocortisol | 14 | 4 | 13 |
| $2\alpha$-Methylcortisol | 0 | 0 | 0 |
| $\Delta^4$-Androstene-3,17-dione | 179 | 171 | 146 |
| Testosterone | 164 | 155 | 83 |
| 19-Nortestosterone | 87 | 101 | 64 |
| $17\alpha$-Methyl-19-nortestosterone | 108 | 105 | 48 |

purified state to be active with a variety of steroid substrates. The relative rates, however, were quite different from those observed in the case of the $5\alpha$ microsomal enzyme (Table II).

*Effect of ovariectomy and hypophysectomy in female rats on levels of microsomal $5\alpha$-enzyme.* The sex differences suggested the possibility that the gonads may be involved in control of $\Delta^4$ reducing enzyme activity in rat liver. Accordingly, female rats were ovariectomized at 21 days of age and when examined 28 and 33 days later showed only a slight but consistent drop of 10% in titer of the $5\alpha$ microsomal enzyme. Animals injected subcutaneously with a daily dose of 50 $\mu g$ of estradiol-$17\beta$ benzoate showed no change from control values. Hypophysectomized female rats (140-150 g initial body weight) showed a 73% drop in reducing activity 10 days after operation. Thirty days after operation no reducing activity was detectable in the microsomal fraction. Loss in activity was reduced about 50% by administration of ACTH (1.0 I.U./day), pregnant mare serum (10 I.U./day) or growth hormone (Horner, 10 $\mu g$/day) for 10 days.

*Variations in microsomal $5\alpha$-enzyme with age in female rats.* Striking differences were noted in the $5\alpha$ reducing activity in livers from female rats of different age groups ranging from 10 days to more than 18 months (Table III). The low enzyme titer in the 10-day-old rats was increased threefold in 15 to 21-day-old animals. Between the 21st and 43rd days of life the enzyme content was again tripled. This high level of activity remained constant at least until the rats were 11 months old. A significant drop, however, was observed in a group of animals older than 18 months.

*Discussion.* There is no explanation at present for the marked qualitative and quantitative differences in concentration of liver $\Delta^4$-hydrogenases between female and male rats. The possibility of a gonadal control of these enzymes, while not excluded, received little support from ovariectomy experiments in the female. Furthermore, a sharp increase in enzyme titer takes place before sexual maturation in the female, which again argues

TABLE III. Variations of $5\alpha$ Microsomal Enzyme Activity in Livers of Female Rats of Different Ages (One Hr Incubation).

| Age (days) | No. of rats | 11-Deoxycortisol reduced (%) |
|---|---|---|
| 10 | 20 | 8 |
| 15 | 18 | 24 |
| 21 | 15 | 26 |
| 27 | 8 | 67 |
| 43 | 2 | 85 |
| 97 | 3 | 88 |
| 330 | 6 | 88 |
| >540 | 3 | 25 |

against a major role for the ovary in control of the 5$\alpha$ enzyme level. The more marked effects of hypophysectomy are difficult to interpret, especially as several trophic hormones were able in some degree to restore the levels of activity toward normal.

*Summary.* 1. The rate of $\Delta^4$ reduction of 11-deoxycortisol was 3-4 fold greater in female than in male rat liver whole homogenates. The same sex difference was found in microsomal fractions containing the $\Delta^4$-5$\alpha$-hydrogenase. 2. Evidence was found for only one $\Delta^4$-hydrogenase (5$\alpha$-microsomal) in female rat liver in contrast to the male liver which contains the soluble $\Delta^4$-5$\beta$-hydrogenase as well. 3. Ovariectomy of adult female rats did not produce any marked change in enzyme titer. Hypophysectomy resulted in a sharp drop which could be reversed in part by ACTH, growth hormone or pregnant mare's serum. 4. Rapid increases in the titer of $\Delta^4$-5$\alpha$-hydrogenase in the liver of the female rat occurred prior to the known stages of sexual maturation.

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## Reversal of Hepatic Venous Circulation in Dogs.* (24431)

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Much has been learned over the years about the function of liver in animals by surgically altering its normal vasculature. Ligation of the hepatic artery(1), Eck fistula(2), transposition of portal vein and vena cava(3), and partial occlusion of the hepatic outflow tracts (4) have all contributed to a better understanding of the complexities of hepatic function. Several years ago one of us (CGC) conceived that additional information about function and hemodynamics of the liver might be gained were it possible to study for prolonged periods animals whose hepatic venous circulation had been reversed. This report describes experiments in dogs as a result of which all caval blood caudad to the diaphragm enters the liver through the hepatic veins and leaves, together with the hepatic arterial blood, by way of the portal vein. Dogs with this type of reversal of hepatic blood flow have now survived up to 57 days in apparent good health. Didier(5) demonstrated in dogs that the flow of portal blood alone could be reversed for a few hours. He did his experiments at the suggestion of M. Hallion, but did not, as far as we have been able to determine, secure prolonged survival among his animals. Didier's experiments were not known to us when we started our investigations of reversal of hepatic venous circulation.

*Materials and methods.* Healthy mongrel dogs weighing 12 to 44 kg were anesthetized with intravenous pentobarbital sodium, 60 mg/2.2 kg of body weight. Animals were maintained during open thoracotomy by intermittent positive pressure endotracheal insufflation. A crimped teflon† vascular prosthesis, 1 cm internal diameter and varying in length with size of dog from 11 to 15 cm, was used to maintain free flow of blood from the side of the superior mesenteric vein to the side of thoracic inferior vena cava. After completion of end to side anastomoses, 50 mg of heparin in 500 ml of isotonic saline solution was administered slowly over 3 hours into a convenient vein in the jejunal mesentery. When the vascular prosthesis between superior mes-

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† C. R. Bard, Summit, N. J.

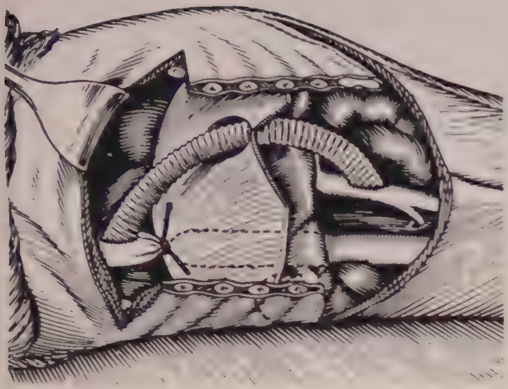
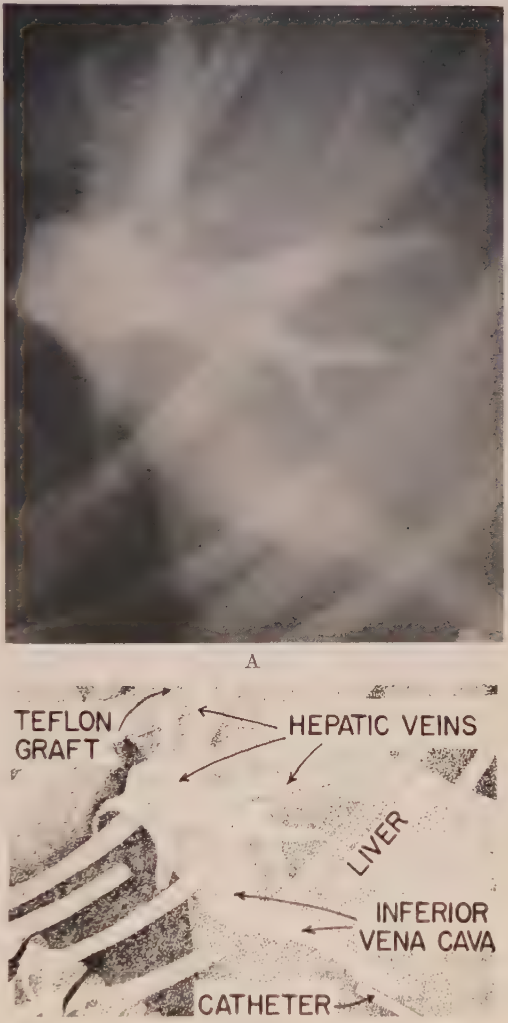


FIG. 1. Diagrammatic representation of reversal of hepatic venous circulation in the dog, right upper abdomen, right lower thorax and right diaphragm are exposed. Free blood flow from superior mesenteric vein to inferior thoracic vena cava is provided by crimped teflon vascular prosthesis sutured in end-to-side positions. The vena cava has been securely ligated with heavy braided silk just above diaphragm. All caval blood, to and including diaphragmatic veins, enters liver through the hepatic veins and leaves the organ through the portal vein. It is joined at sinusoidal level by the hepatic arterial blood and at about site of superior mesenteric anastomosis by splanchnic venous drainage. Preparations of this type are now surviving 2 mo after operation in apparent good health.

enteric vein and inferior thoracic vena cava appeared to be functioning nicely, the vena cava was securely ligated, and in many dogs divided, just cephalad to diaphragm (Fig. 1). The abdominal and thoracic wounds were then closed in layers. During operation unmatched citrated whole dogs' blood was administered when necessary. On the day following operation and thereafter the animals were allowed free access to water and a standard laboratory diet consisting of dog meal and canned horse meat. Seven and 30 days following operation cinefluroscopic and venographic studies were performed upon 3 survivors. In each instance 20 ml of 70% Diodrast solution was injected into the inferior vena cava just below entrance of the renal veins. The opaque mass could be followed radiographically to the sinusoidal level within the liver. Here, free mixing with hepatic arterial blood precluded visualization of portal venous radicals. The hepatic shadow could be, however, identified. As caval and hepatic arterial blood left the liver, its Diodrast content was further diluted by mixing at site of portal anastomosis with splanchnic portal blood. Nevertheless, visualization of the vascular prosthesis was obtained. A representative caval venogram and hepatogram is reproduced in Fig. 2, together with an explanatory diagram. Microscopic sections were made of small segments of liver removed from some animals on 4th, 5th, 42nd, and 50th days after operation. The only microscopic features of these livers, distinguishing them from



A

B

FIG. 2A and 2B. Caval-hepatic venogram, following reversal of hepatic venous circulation.

FIG. 2A. Unretouched cavalgram and hepatic venogram obtained by inj. 20 ml of 70% Diodrast into vena cava of dog just caudad to entrance of renal veins. The contrast medium can clearly be seen in the vena cava, in hepatic veins, in liver and faintly in teflon vascular prosthesis.

FIG. 2B. Tracing of roentgenogram (A) appropriately labeled.

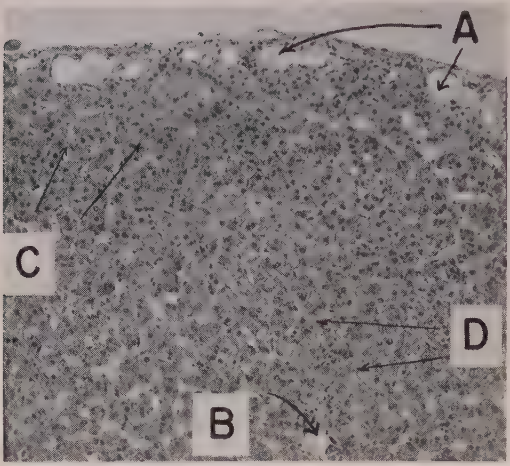


FIG. 3. Microscopic sections obtained from dog whose hepatic venous circulation had been reversed for 47 days. A = dilated subcapsular lymphatics; B = dilated central veins; C = sinusoidal congestion; D = central necrosis.

normal, are: (1) dilated subcapsular lymphatics; (2) dilated central veins; (3) sinusoidal congestion; and (4) small areas of central necrosis of hepatic cells. Representative microscopic section is reproduced in Fig. 3. Total protein, serum albumin, serum globulin, serum bilirubin, blood ammonia, bromsulfalein excretion, and serum glutamic oxaloacetic transaminase were measured at intervals after operation in the peripheral blood of surviving animals. The only consistent abnormality was a persistently elevated blood ammonia in most animals.

*Results.* Hepatic venous circulation has been reversed in 19 dogs. Seven have survived 4, 5, 17, 23, 27, 47, and 57 days after operation. One healthy survivor died during an exploratory celiotomy performed 47 days after operation. The cause of death was clearly pulmonary aspiration of gastric contents. The remaining 12 animals died within 6 to 18 hours of operation. In 8 the teflon prosthesis and both anastomoses were patent; in 4 these were partially compromised by thrombi. The cause of this high postoperative mortality has been obscure. At one time we thought it might relate to inactivation of cortical adrenal steroids by their immediate passage through the liver(6). Cortisone acetate administered before and after operation failed, however, to alter our rate of survival.

The abdominal cavities of 2 animals were explored at 42 and 47 days respectively. Each contained several hundred ml of ascitic fluid. The liver in each was enlarged, tense, and dark red in color with areas of blue mottling. The portal pressures were 18 and 20 cm of saline. The peritoneal cavities of 2 animals which died at 4 and 5 days contained 700 to 800 ml of ascitic fluid. The composition of the fluid in these 4 animals was essentially the same as that produced in dogs by partial constriction of the thoracic inferior vena cava with a metal band.

Dogs surviving this operation from 17 to 57 days have all lost up to 30% of preoperative body weight. In this respect they resemble animals with end to side portacaval shunts (Eck fistulae). Whether they will exhibit further nutritional deterioration, "meat intoxication" and death, characteristic of animals with an Eck fistula, is currently under investigation(7).

*Conclusions.* 1. Dogs survive for prolonged periods, at least 57 days, following reversal of their hepatic venous circulation. 2 Crimped teflon vascular prostheses remain patent in these low pressure venous systems and function satisfactorily enough to achieve objectives of this experiment. 3. This experimental preparation is presented as one potentially capable of contributing materially to studies of hepatic function and intrahepatic and extrahepatic vascular hemodynamics.

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# Response of $CO_2$-Deficient Human Cells *in vitro* to Normal Cell Extracts.*

(24432)

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Human cells of HeLa and conjunctival strains failed to show a net increase in numbers when grown in absence of $CO_2$(1). Under conditions of the experiments, cells so arrested for as long as 14 days resumed multiplication when $CO_2$ was again supplied. Grossly, the cells appeared healthy, and active metabolism of radioactive glucose was found. These characteristics, together with resumption of multiplication when $CO_2$ was provided, indicated that cell viability was high and suggested depletion of factors concerned with multiplication rather than general interference with cell permeability or overall $CO_2$-fixation reactions. Experiments herein reported show that depleted HeLa and conjunctival cells can resume growth in absence of $CO_2$ when provided with normal cell extracts. Extracts of depleted cells were ineffective.

*Methods.* Stocks of HeLa and conjunctival cells derived from human carcinoma and human conjunctiva, respectively,† were maintained in Eagle's medium containing 10% horse serum inactivated by heating at 56°C for ½ hour. Preparation of roller tube cultures, determination of cell count, and method of making tubes $CO_2$-deficient have been described(1,2). With $CO_2$-deficient cells, the sole buffer was 1 mM phosphate buffer which sufficed for adequate pH control. Bicarbonate buffer was present in control tubes. Normal cell extract‡ was prepared as follows: stock bottles of cells were grown in normal manner and when a total of approximately 3-4 x $10^6$ cells were present, the medium was removed as completely as possible and the following procedure was employed: 10 ml of Eagle's medium (containing no serum, $MgCl_2$ or $CaCl_2$), was added and the cells incubated overnight.§ Any cell debris remaining on glass was scraped off, and contents of bottle transferred to sterile ampule, sealed, and stored at –70°C until used. Prior to being added to roller tubes, the extract was reconstituted by addition of dialyzed horse serum, $CaCl_2$, and $MgCl_2$. Such media were agitated at 35°C in the presence of a KOH ampule(1) for 4 to 5 hours to remove traces of $CO_2$, then transferred to appropriate roller tube cell cultures with a minimum of exposure to air. When used with $HCO_3^-$-deficient cells, the KOH-containing ampule was always present in the tube. Ampules in these and other $CO_2$-deficient tubes were changed frequently to assure adequate $CO_2$-absorption. In some instances, extracts were prepared from stock cells which were $CO_2$-deficient for 48 hours. Media were changed every other day and all incubations were done at 35°C. All media employed were kept in containers provided with KOH ampules to minimize $CO_2$ contamination in so far as possible. Routine assay experiments were done as follows: freshly prepared roller tube cultures were allowed to incubate 3 days, then were made $CO_2$-deficient for 48 hours. Test material was introduced and incubation continued for 7 days, at which time cell counts were done. Appro-

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† Authors are indebted to Dr. R. S. Chang of Dept. of Microbiology for providing clone cell stocks of both these strains, and also to Dr. Edward Murray, for checking cells for freedom from contamination by pleuropneumonia-like organisms.

‡ The term "extract" denotes the product which results from disintegration of cells by the method described and from which particulate material has not been removed.

§ In absence of $Mg^{++}$ and $Ca^{++}$, these cells rapidly disintegrate(3). This procedure was adopted because manipulation of sterile materials was minimized while still insuring destruction of the cells. On occasion, other methods were used, such as scraping cells into water or medium, followed by lyophylization, and subsequent reconstitution prior to use. These extracts were as active as those made by procedure outlined in text. Furthermore, lyophylization of the usual extract did not impair its effectiveness.

TABLE I. Effect of Cell Extract on Multiplication of Normal and $CO_2$-Deficient Human Cells *In Vitro*.

| Exp. No. | Test cells* | Extract added† | No. cells/tube × $10^3$‡ |
|---|---|---|---|
| | Conjunctival cells | | |
| 1 | Normal | None | 369 (292-486) |
| | Deficient | ” | 199 (160-230) |
| | ” | Normal | 647 (596-700) |
| 2 | Normal | None | 394 (360-422) |
| | ” | Normal | 395 (386-400) |
| | Deficient | None | 172 (156-186) |
| | ” | Normal | 297 (236-362) |
| 3 | Normal | None | 284 (232-328) |
| | Deficient | ” | 77 ( 50- 98) |
| | ” | Normal | 183 (170-196) |
| | ” | Deficient | 32 ( 22- 42) |
| | HeLa cells | | |
| 4 | Normal | None | 291 (192-382) |
| | ” | Normal | 301 (280-314) |
| | Deficient | None | 76 ( 62- 82) |
| | ” | Normal | 314 (220-410) |
| 5 | Normal | None | 264 (210-292) |
| | Deficient | ” | 26 ( 20- 36) |
| | ” | Normal | 201 (178-242) |
| 6 | Normal | None | 144 (124-162) |
| | Deficient | ” | 25 ( 20- 34) |
| | ” | Normal | 48 ( 42- 58) |
| 7 | Normal | None | 228 (200-270) |
| | Deficient | ” | 81 ( 56-128) |
| | ” | Normal | 174 (158-200) |
| | ” | Deficient | 29 ( 21- 40) |
| 8 | Normal | None | 695 (640-768) |
| | Deficient | ” | 69 ( 60- 78) |
| | ” | Normal | 301 (260-378) |
| | ” | Deficient | 20 ( 4- 48) |
| 9 | Normal | None | 178 (161-194) |
| | ” | Normal | 191 (172-211) |
| | Deficient | None | 45 ( 44- 47) |
| | ” | Normal | 245 (198-288) |

* In Exp. 1 and 2, cells were allowed to grow for 9 and 18 days, respectively, prior to removal of $CO_2$. Subsequent treatment was as given in the text.

† Conjunctival cell extracts were used in Exp. 1-3, and HeLa cell extracts in Exp. 4-9. Amount of extract was that derived from approximately 3 × $10^5$ cells.

‡ Expressed as avg and range.

priate sets of control tubes were included. The procedure adopted penalized the deficient cells grown with extract because their growth would have been arrested during the 48 hours of depletion, whereas that of non-deficient cells was not. In experiments where this discrepancy was taken into account, the extract-treated deficient cells always showed at least normal multiplication. However, from the standpoint of the immediate problem, the important point was whether or not multiplication occurred. Where radioactive acetate was employed, the procedures have been previously described (4). Additional pertinent data are given in footnotes of the appropriate Table.

*Results.* Both human HeLa and conjunctiva cells multiplied in the absence of $CO_2$ when normal cell extracts were present (Table I). No stimulation of multiplication of non-deficient cells was caused by such extracts. From Fig. 1 it is apparent that the amount of extract employed in the usual assay was well within the optimum range. Material in approximately 4 to 5 normal cells was sufficient to cause one deficient cell to resume multiplication. Extracts from $CO_2$-deficient cells not only failed to stimulate multiplication of deficient cells (Fig. 1), but actually caused reduction in cell count. This suggests that the deficiency may have been made more severe by such extracts.

Since depleted cells respond to added $CO_2$ (1), care was exercised to eliminate this as a possible contaminant. Use of KOH ampules is highly effective in this regard (1). As determined by use of $NaHC^{14}O_2$, less than 0.02% of normal $CO_2$ and $HCO_3^-$ is found in the presence of the ampule. Contamination of cell extracts by viable cells is unlikely because extract added to control tubes caused no increase in cell count (Table I), nor have cells

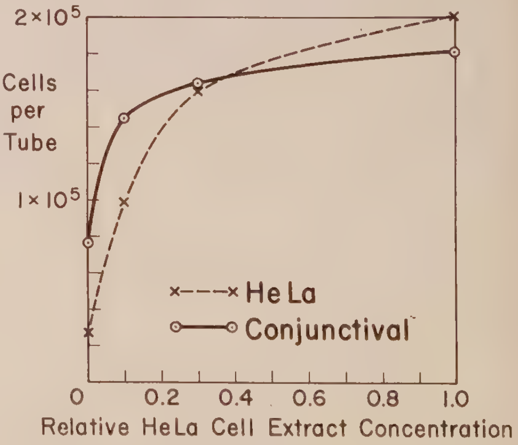

FIG. 1. Effect of concentration of normal HeLa cell extract on the multiplication of $CO_2$-deficient cells. A cell extract conc. of 1 is equivalent to material contained in approximately 3 × $10^5$ cells made up to a final vol of 1 ml with Eagle's medium. Each point represents avg of 4 results.

TABLE II. Conversion of Acetate-1-$C^{14}$ to $C^{14}O_2$ by Normal and $CO_2$-Deficient HeLa Cells.*

| Kind of cells | Cell count × $10^3$ | Total conversion to $C^{14}O_2$ (c.p.m. × $10^3$) | Relative conversion to $C^{14}O_2$ (c.p.m./$10^3$ cells) |
|---|---|---|---|
| Normal | 177 (161-194) | 27 (26-30) | 152 |
| $CO_2$-deficient | 45 ( 43- 49) | 12 ( 9-13) | 266 |

* These results pertain to cells which were 8 days old from time of inoculation. $CO_2$-deficient cells were without $CO_2$ for last 5 days of this period. On the seventh day, all tubes received acetate-1-$C^{14}$ to furnish 1.38 × $10^6$ counts/min. (specific activity = 15 millicuries/millimole acid). Values are mean of 4 tubes; numbers in parentheses give range.

ever grown out of the reconstituted extracts even after 3 weeks' incubation.

*Discussion.* Of considerable interest is the fact that whereas within 24 hours after removal of $CO_2$, net multiplication has ceased (1), metabolism of acetate-1-$C^{14}$ to $C^{14}O_2$ is approximately normal even at the end of 5 days (Table II). Earlier(1), glucose-1-$C^{14}$ conversion to $C^{14}O_2$ by such cells was found to be two-thirds normal after 2 days' depletion. Therefore, inhibition of net multiplication is one of the first effects of $CO_2$-depletion and is not accompanied necessarily by generally deranged metabolism. This is not to say that other reactions involving incorporation of $CO_2$ are necessarily normal or adequate in the depleted cells. Interference with nucleic acid synthesis would afford ample cause for inhibition of multiplication. Such a possibility is now under investigation. The importance of $CO_2$ in living cells has been pointed out by Loomis(5) and Werkman(6). The former has laid special stress on $pCO_2$ as a major regulatory agent in the control of biological function. He suggests $CO_2$ is the agent involved in such studies as those of Clowes(7) and Cook and Elvidge(8) on sperm stimulation and those of Puck(9) on a diffusable factor in tissue culture The manner in which $CO_2$ would function in such cases is not discussed. From the present results, it is apparent that $CO_2$ can be replaced by cellular material synthesized by normal cells with an adequate $CO_2$ supply. It is also possible that similar results would be found in other cases where $CO_2$ has been shown to be essential. Elucidation of the active agent(s) should afford further insight into control of cell multiplication. The earlier finding of Harris(10) that $CO_2$ was essential for the outgrowth of chick heart fibroblasts suggests that the phenomena observed here are not restricted only to cells which have been maintained in continuous culture for long periods.

*Summary.* Human cells of HeLa and conjunctival (Chang) strains fail to multiply in absence of $CO_2$, but net multiplication can be restored by addition of normal cell extract. Such extracts have no effect on multiplication of normal cells. On the other hand, extracts of $CO_2$-deficient cells have an adverse effect on deficient cells. Although net multiplication was completely arrested in the absence of $CO_2$, conversion of acetate-1-$C^{14}$ to $C^{14}O_2$ was at least normal, indicating general cell metabolism was still high. Implications of the results to regulation of cell multiplication are discussed.

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# Immunologic Tolerance in Rats to Type Specific Antigens of Human Erythrocytes.*† (24433)

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Reduced ability of an animal, exposed early in life to a specific foreign antigen, to react immunologically to the same antigen in later life is known as "actively acquired tolerance"(1). Most studies using erythrocytes as antigen have involved identical or closely related species(2,3). The possibility of producing tolerance to antigens of erythrocytes of distant species has been investigated by injecting red cells from rabbits into chicks(4), sheep into rats(5) and humans into birds(6), all with negative results. However, other investigators have reported depression or delay of antibody formation by injecting human erythrocytes into rats, rabbits and chicks(7, 8). The feasibility of inducing tolerance to human blood antigens is relevant to bone marrow transplantation studies and conditions involving blood group incompatibility. It was the purpose of this investigation to determine whether human erythrocytes could confer type specific immunologic tolerance when injected into prenatal or neonatal rats.

*Material and methods.* Twenty litters of rats of the Sprague-Dawley strain were used. Seven of 8 litters injected pre-natally at laparotomy were lost at birth and one additional litter injected at birth died in immediate post-natal period. Preliminary studies on erythrocytes from 50 adult rats of this strain revealed, in all instances, strong agglutination of these cells by commercial human anti-B serum and no agglutination with Anti-$A_1$ or anti-D ($Rh_o$) serum. Human erythrocytes of types A, Rh-pos. (A+), A, Rh-neg. (A–) or O, Rh-neg. (O–), stored in Alsever's solution, were washed in saline and used as antigen. To induce tolerance, one intraperitoneal injection of 0.2-0.4 cc of a 50% erythrocyte suspension was given during the peri-natal period. Later, immunization injections consisting of 0.5 cc of a 50% A+ erythrocyte suspension were administered subcutaneously. Five litters totaling 38 rats (Table II) received a single immunizing injection, another 5 litters totaling 44 rats (Table I) received 7 injections on alternate days over a period of 2 weeks. Two-fold dilutions of inactivated serum (56°C for 30 minutes) were prepared in 0.1 ml amounts. To this an equal amount of a 2% suspension of washed A+ erythrocytes in .15M saline was added. The tubes remained at room temperature (24 ± 4° C) for 20 minutes and centrifuged at 200 G for one minute. A reading of 1+ agglutination was considered the end point. Agglutinin titers of rat sera with human A+ cells are expressed as $\log_2$ of the reciprocal of the final dilutions. A difference of 2 or more titer units (equivalent to a four-fold dilution) was considered meaningful. Wherever possible erythrocytes from the same individual were used for injections and titrations. Tests for "incomplete antibodies" were carried out in the usual manner employing both 22% bovine albumin as protein diluent and chicken anti-rat globulin serum. Absorption experiments were performed at room temperature (24 ± 4°C) by adding 2 successive volumes of 0.2 ml of packed O– human erythrocytes, washed in .15 M saline, to 0.4 ml of test serum. All the rats were weighed at weekly intervals. No difference was observed between those receiving and those not receiving the "tolerance" injection.

*Results.* Table I and Fig. 1 show the findings for the group of animals receiving multiple immunization injections over a 2-week period. Animals which had received a "tolerance" injection of A+ erythrocytes during the peri-natal period and challenged as late as 83 days after birth showed appreciably lower titers than the animals which had not received a "tolerance" injection. Injection at birth of O– erythrocytes (litter T-6) appar-

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TABLE I. Animals Receiving Immunizing Injections on Alternate Days over a Period of 2 Weeks.

| Litter No. | No. of animals | Age at tolerance inj. | RBC's inj. at tolerance inj. | Age at immunization with A+ RBC's (days) | Avg unabsorbed titers against A+ erythrocytes (reciprocal of dilutions expressed as $\log_2$)* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Immed. before immunization | Wk after final immunizing inj. | | | | | | |
| | | | | | | 0 | 2 | 3 | 4 | 5 | 8 | 10 |
| T-7 | 4 | 3 days | A+ | 40 | .8 | 6.5 | | | | 8.8 | 7.5 | 7.3 |
| | 2 | | None | 40 | 1.5 | 12.0 | | | | 12.0 | 12.0 | 13.5 |
| | 2 | 3 days | A+ | 100 | 3.0 | 9.5 | | 9.0 | | | | |
| | 2 | | None | 100 | 2.5 | 11.5 | | 12.5 | | | | |
| 4 | 7 | 3 days before birth | A+ | 83 | 1.0 | 7.3 | | | 9.4 | | | 8.6 |
| 6 | 7 | At birth | O– | 68 | .6 | 10.7 | | 11.0 | | | 10.3 | |
| 8 | 8 | 4 days | A+ | 65 | 1.5 | 7.1 | 9.3 | | | | | 9.1 |
| | 3 | | None | 65 | .0 | 10.3 | 11.7 | | | | | 11.0 |
| 12 | 5 | At birth | A+ | 30 | .8 | 7.2 | 9.6 | | | | 9.6 | |
| | 4 | | None | 30 | .5 | 10.5 | 11.3 | | | | 11.5 | |

* The difference of 2 or more titer units (a 4-fold dilution) was considered meaningful.

ently had little or no effect on later immunization with A+ cells. An apparent loss of tolerance with time is illustrated by comparing the response of the animals immunized at 40 days with their litter-mates immunized at 100 days (litter T-7).

Table II and Fig. 2 demonstrate a similar inhibition of agglutinin response for the tolerant animals which received a single immunizing injection. Again injection of O– cells at birth conferred no immediate protection to later immunization but the response obtained was less sustained than in the controls. The results observed in litters T-14 and T-17 (Table II) seem to indicate that absence of the D ($Rh_o$) antigen in the initial injection reduced degree of tolerance obtained.

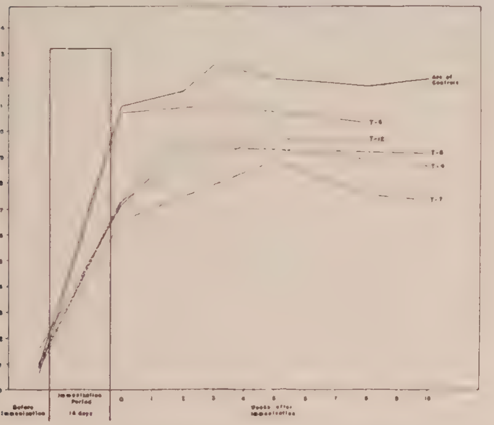

FIG. 1. Avg unabsorbed titers (reciprocal of dilution expressed as $\log_2$) against A+ erythrocytes for rats receiving immunizing inj. on alternate days over a period of 2 wk. Interrupted lines represent values for rats which received "tolerance" inj. of A+ cells in early life. Rats in T-6 received O– erythrocytes at birth. Controls were given no inj. in perinatal period. (See Table I for details.)

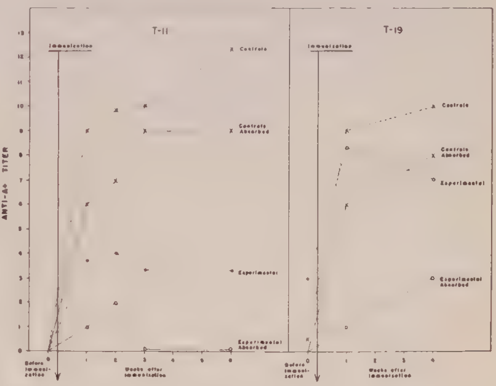

FIG. 2. Titers expressed as in Fig. 1. All rats received one immunization inj. at arrow. Experimental animals were inj. in neonatal period with A+ erythrocytes; controls did not receive this inj. Interrupted lines signify avg unabsorbed titers; solid lines represent titers obtained with same sera after absorption with O– human erythrocytes.

It should be emphasized that ability to induce tolerance in rats to human erythrocytes is by no means a uniform phenomenon. Marked differences were observed among individual litter-mates and between litters. This has been reported in a similar system(7) and was observed also for mouse skin homografts (1).

In 2 other litters (T-10 and T-20) the initial "tolerance" injection of A+ erythrocytes was delayed until 2 weeks after birth. All 10

TABLE II. Animals Receiving Single Immunizing Injection.

| | | | | | Avg unabsorbed titers against A+ erythrocytes (reciprocal of dilutions expressed as $\log_2$)* | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Wk after immunizing inj. | | | | |
| Litter No. | No. of animals | Age at tolerance inj. | RBC's inj. at tolerance inj. | Age at immunization with A+ RBC's (days) | Immed. before immunization | 1 | 2 | 3 | 4 | 6 |
| T-11 | 3 | At birth | A+ | 30 | 0 | 3.7 | 4.0 | 3.3 | | 3.3 |
| | 4 | | None | 30 | 0 | 9.0 | 9.8 | 10.0 | | 12.3 |
| 14 | 4 | 1 day | A+ | 80 | 0 | 4.3 | | 8.0 | | |
| | 3 | " | A− | 80 | 0 | 7.0 | | 8.2 | | |
| 17 | 6 | At birth | A+ | 73 | | 6.2 | | 5.8 | | |
| | 6 | " | A− | 73 | | 7.2 | | 7.7 | | |
| 16 | 7 | " | O− | 67 | .4 | 10.0 | | | 8.4 | |
| 19 | 3 | 1 day | A+ | 56 | 3.0 | 8.3 | | | 7.0 | |
| | 2 | | None | 56 | .5 | 9.0 | | | 10.0 | |

* The difference of 2 or more titer units (a 4-fold dilution) was considered meaningful.

injected animals showed an immune response.

To investigate the possibility that the apparent depression of immune response may have been due to formation of antibodies other than saline agglutinins(7), tests for "incomplete antibody" were performed. No evidence for the presence of "incomplete antibodies" was obtained.

Absorption with fresh human O− erythrocytes reduced the antibody titer against A+ cells in the non-tolerant animals. In similarly absorbed sera from tolerant rats, agglutinins against A+ cells were either markedly reduced or not demonstrable (Fig. 2). Thus, it appears that another antigen or group of antigens probably related to species differences, was responsible for most of the antibody observed in the tolerant animals. Very early embryonic injections may be required to produce complete tolerance to these species-specific factors. It is not unlikely that some of the inconsistencies reported in experiments dealing with wide species differences may be explained on this basis, although other variables such as dose or route of injection, age of animals at tolerance or immunization injections, and at time of bleeding for serum, undoubtedly play an important role.

*Summary and conclusions.* Intraperitoneal injection of human erythrocytes in pre-natal or neo-natal rats reduces antibody response to subsequent immunizations. No ill-effects were observed in any of the experimental animals surviving the initial injection and there was no difference in growth rate from the uninjected controls. These studies suggest that it is possible to confer type specific tolerance to subsequent immunization of A and D ($Rh_o$) antigens of human erythrocytes.

The authors are grateful for the helpful suggestions offered by Prof. Ray D. Owen in reviewing our data, and by Dr. William H. Stone in reviewing the manuscript. We also wish to thank Mrs. Delle Flakas for her technical assistance.

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# Autoradiographic Studies of Sulfated Mucopolysaccharide Metabolism in Cartilage of Osteolathyric Rats and Chicks.* (24434)

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Experimental osteolathyrism is produced by feeding animals seeds of *Lathyrus odoratus*(1, 2) or injecting them with the toxic factor $\beta$-aminopropionitrile(3). One of the characteristic features of this syndrome is hypertrophy of cartilage(1,2) accompanied by disorganization of the cellular architecture(2,3,4) formation of clefts(4), slipping of articular surfaces (2,3,4,5) and detachment of tendinous and ligamentous insertions(2). These conditions have been theoretically ascribed to "defective formation or excessive destruction of chondroitin sulfate of ground substance"(2). Sulfur$^{35}$ recovered chemically from skeleton, has been increased after 7 days(6) in animals when $P^{32}$ of bone was also increased. At the same time, weight of femurs was increased while general body weight was smaller than that of controls(6). Autoradiographic studies made 20 hr after injection of tracer sulfate have shown "deposition of $S^{35}$ in about equal amounts in experimental as well as in control animals"(7). On the other hand, histochemical studies involving use of periodic acid-Schiff, alcian blue and toluidine blue have led Menzies and Mills to conclude that when sweet pea seed was fed to rats, it produced in the aorta and bone, "an accumulation of chondroitin sulfate with the general breakdown of supporting structures such as collagen fibers" (8). Our experiments have been carried out on 2 groups of 6 weanling Sprague-Dawley rats fed a 50-50 mixture of complete diet and *Lathyrus odoratus* seed ground thin, for 10 and 16 days with the same number of controls fed the complete diet; also 6 one-day-old chicks which ate a 50-50 mixture of Purina Growing Mash and ground Lathyrus seed and were kept for 7 days at 35°C along with 6 controls on Growing Mash exclusively. Some rats after 16 days of lathyrus feeding and most chicks after 7 days showed symptoms of severe intoxication(5).

*Methods.* All rats and 3 chicks in each group were injected with 1 $\mu$c/g $S^{35}O_4$ subcutaneously, 2 hr prior to decapitation. The tibiae were demineralized with 5% nitric acid in 70% ethanol for 7 days, then dehydrated and embedded in paraffin. Sections of tibiae of chicks, which had not been injected with radiosulfate, were placed for 1 hr in a weak solution of $Ca^{45}Cl_2$(9), then thoroughly washed, dehydrated and prepared for autoradiography by the coating-inversion technic (10,11) along with similar sections from animals injected with $S^{35}$.

*Results.* In control rats (Fig. 1), the radiosulfate appeared incorporated by small and large cells except in calcifying cartilage next to the site of bone formation (Fig. 1). Cartilage cells were neatly arranged in columns parallel to the bone axis. A small amount of ground substance was visible between the columns of cells. The autoradiographic image was almost exclusively related to the chondrocytes, 2 hours after subcutaneous injection of tracer (Fig. 1).

The epiphyseal plate of the tibia in lathyric rats of 10 days, (Fig. 2) was already thicker than that of controls (Fig. 1). The lower border appeared jagged and columns of chondrocytes were irregularly arranged and dispersed in a more abundant matrix (Fig. 2). A comparable amount of radiosulfate seemed to have been retained by individual chondrocytes (Fig. 2). The radioactive material appeared also almost exclusively intracellular at 2 hrs. It seemed, however, that the increase in thickness of the plate was due to a larger amount of ground substance and also to an increase in number of cells capable of synthetizing radiosulfur (Fig. 2). The thickness of cartilage where no synthesis took place showed less change (Fig. 2).

In rats which had been on the lathyric diet for 16 days, the epiphyseal plate was thicker

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* With technical collaboration of Cécile Bélanger, Céline Lemay and Andrée Boucher. Aided by grants from Nat. Research Council of Canada (Med. Division and Comm. on Dental Research).

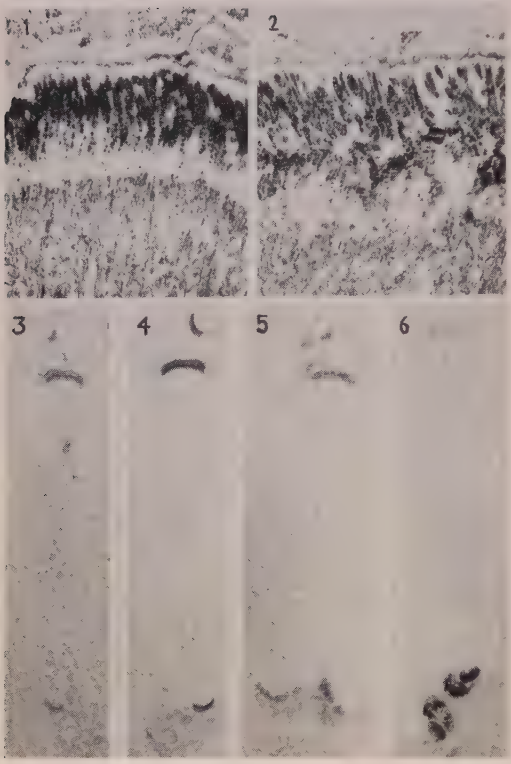

FIG. 1. Integrated, stained autoradiograph of epiphyseal plate of a normal young rat 2 hr after subcut. inj. of radiosulfate, × 26.

FIG. 2. Integrated, stained autoradiograph of epiphyseal plate of a young rat on lathyrogenic diet for 10 days and 2 hr after radiosulfate, × 26.

FIG. 3. Integrated, unstained autoradiograph of tibia of 7-day-old chick, 2 hr after radiosulfate, × 1.25.

FIG. 4. Integrated, unstained autoradiograph of tibia of 7-day-old chick on lathyrogenic diet, 2 hr after radiosulfate, × 1.25.

FIG. 5. Integrated, unstained autoradiograph of tibia of 7-day-old chick, 1 hr *in vitro* treatment with $Ca^{45}$, × 1.25.

FIG. 6. Integrated, unstained autoradiograph of tibia of 7-day-old chick on lathyrogenic diet, 1 hr *in vitro* treatment with $Ca^{45}$, × 1.25.

but there was also a proportional increase in amount of ground substance and of cells capable of picking up $S^{35}$ *in vivo*. These results agree with the observations of Ponseti *et al.*(7) and possibly also with conclusions of Bauer *et al.*(6) in regard to overall sulfur content of the epiphyseal plate since the total number of cells capable of synthetizing sulfate is increased.

Furthermore, localized areas of sulfur uptake in connection with activated callus-like growth at the periosteum were also observed in our lathyric rat autoradiographs. These observations reinforce the opinion of Bauer *et al.*(6) in regard to overall sulfur content in the bone.

Hypertrophic changes observed in the rat were not apparent in the chick after 7 days (Figs. 3, 4, 5, 6). In birds, radioactive sulfur was found in similar locations in both controls (Fig. 3) and lathyric animals (Fig. 4). Autoradiographs have shown this material deposited mainly in a layer of small cells near the junction of articular and growing portions of cartilage and in a second layer of large cells preceding the mineralized portion (Figs. 3, 4). There seemed to be larger uptake at the faster growng extremity (Figs. 3,4) and also an equal or larger uptake by cartilage of lathyric chicks (Fig. 4) as compared to that of normal birds (Fig. 3).

These observations on radioactive sulfur uptake in the live animal are not informative in regard to residual content of the matrix which results from synthetic activity of the chondrocytes(12) but is also dependent on rate of turnover or utilization. *In vitro* uptake of $Ca^{45}$ by demineralized sections of tissue has given results in previous problems(9,13,14) of skeletal physio-pathology. It is based on the demonstrated ability of chondroitin sulfate to act as a cation binder(15). With the present material, the upper extremity of the tibia in normal chicks (Fig. 5) showed a larger amount of $Ca^{45}$ uptake in the growing zone of cartilage as compared to the articular portion. The lower extremity of bone seemed to contain slightly more than the upper extremity; the tibiae of lathyric chicks contained considerably less material capable of binding $Ca^{45}$ *in vitro* (Fig. 6) and by opposition, the lower extremity contained a much larger amount (Fig. 6). It is noteworthy that in animals which have shown cleavage and slipping of the epiphysis(5), the upper extremity of the tibia was always involved while the lower extremity remained intact.

These facts are interpreted as showing a difference in metabolism and growth activity of the 2 extremities of the tibia. Even if lathyric animals have a normal or slightly exaggerated uptake of sulfate probably incorporated as chondroitin sulfate(16,17), the turnover or loss of that substance must be greater at the upper extremity of bone so that the residual content is less than that of controls after 7 days. On the other hand, the lower extremity, with a much slower growth potential, seems to have accumulated more cation binder than that of the normal chick.

In previous studies, the hypertrophic epiphyseal plate of rickets(9,14) and fluorosis (13) has shown an accumulation of cation binder, while with impaired synthesis, the epiphyseal plate of magnesium deficient rats has shown a lower residual content(18).

*Summary.* In lathyric rats and chicks, the *in vivo* sulfate fixation per individual cell was equal or slightly higher than that of normal animals. Since there were more active cells in the hypertrophic plate of the lathyric rat, the overall sulfur upake by cartilage was apparently greater. In 7 day lathyric chicks, the plate was not hypertrophied but the sulfate uptake seemed slightly greater. *In vitro* $Ca^{45}$ uptake by demineralized cartilage of the upper extremity of the tibia was considerably less than that of the lower extremity and also less than that of controls, indicating a more rapid utilization or turnover.†

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† PROC. SOC. EXP. BIOL. AND MED. (v98, 318) contains a report by Castellani and Castellani-Bisi showing that in a mixture of epiphyseal plates from osteolathyric rats, hexosamine was significantly decreased (36%) while hydroxyproline remained practically unchanged.

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## Factors Influencing Isoimmunization in Rabbit. Effect of Early Postnatal Immunization on Specific Isoantibody Response.* (24435)

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Although our knowledge of blood groups, incompatibility, and hemolytic disease has increased considerably in recent years, the fact that only about 5% of Rh-negative women become immunized during pregnancies in which the necessary preconditions for Rh incompatibility exist has not been fully explained(1). Mitchison, Brambell(1) and

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* This study was carried out at Roscoe B. Jackson Memorial Lab., Bar Harbor, Me., and supported by grants from Nat. Inst. of Neurol. Dis. and Blindness, Bethesda, Md.

Owen(2) have suggested that the probability of isoimmunization may be dependent on a form of immunological tolerance which is predicted by the "self marker" theory of Burnet and Fenner(3). On this basis the Rh-negative daughter of an Rh-positive mother should be unable to produce Rh antibody if she is carrying an Rh-positive child. Booth *et al.* (4) could not corroborate this explanation by their data. However, Owen(2) has suggested that the explanation on the basis of "tolerance" is still useful, since the effect is represented by a difference in amount or kind of antibody produced even if an absolute form of tolerance does not exist. Since Billingham *et al.*(5) demonstrated *actively acquired tolerance* to skin grafts in mice and chicks, many attempts have been made to induce tolerance-like phenomena with all types of antigens in mice, chicks, rabbits, and rats(6,7,8,9). It has been implied in some of these studies that true acquired tolerance is most effective in cases where there is a close relationship between the origin of antigenic material and the recipient animal. A number of investigators have reported that injection of large amounts of defined soluble antigens into newborn rabbits leads to persistent specific inhibition of antibody production(10,11,12,13,14). In the present study we tried to influence the amount of isoantibody produced in adult rabbits by introducing incompatible blood into the newborn rabbit. This represents the first systematic testing of the hypothesis of Mitchison, Brambell, and Owen that inhibition of antibody production may result from exposure of the fetus or newborn to incompatible homologous blood.

*Materials and methods.* Rabbits were of mixed genetic stocks produced at Jackson Laboratory and were selected as donors and mates solely on the basis of blood type. Immune testing sera were prepared by isoimmunization(15), and all animals were blood typed for the 8 specific erythrocytic antigens which are controlled by 5 genetic loci(16). We were particularly concerned with the antigens belonging to the one genetic system for which we have accumulated data on the probability of isoantibody production. This blood group system has 3 alleles, each of which gives rise to a detectable antigen (A, D, or F). We were able, therefore, to determine the complete genotype of each animal used. Matings were made so that all offspring in a particular litter lacked the donor antigen (type A red cells). *Injection schedules*: Newborn animals were divided into 2 groups; one was injected intraperitoneally 15 times, starting at birth, with 0.5 ml of incompatible citrated blood, and the other (controls) received no early postnatal injections. After the first 15 injections, treated animals were separated into 3 subgroups. One subgroup received 2 intraperitoneal injections/week of incompatible blood until the age of 4 months. Another subgroup received one injection/week of incompatible blood until reaching approximately 3½ months age. A third subgroup received no injections following the first 15 injections. At age 4 to 5 months, all animals were subjected to our regular isoimmunization program, which consisted of injection of incompatible red blood cells in a modified Freund's adjuvant given intermuscularly on 2 occasions followed by a series of intravenous injections of glycerine-citrate-treated red cells. The animals were bled one week after the 5 intravenous injections, which were given over a 2-week period. They were then subjected to another course of 6 intravenous injections over a 2-week period and were bled again one week after last injections. *Antigens.* The initial 15 injections, as well as those given before beginning our regular immunization procedure, were of sterile rabbit blood in ACD solution; proportion of whole blood to ACD solution was 3:1. The antigen used for injection in our standard immunization procedure consisted of washed red cells preserved at –4°C in a glycerine-citrate mixture(17). The cell suspension was thawed and injected without removing the glycerine. At no time did we see any unusual reaction to the injection of the glycerine-citrate red cell mixture. *Titrations.* Trypsin-treated cells were used for titration against sera made up in serial doubling dilutions. The serum-cell mixtures were read for agglutination and strength of reactions was scaled according to a modification of scoring system suggested by Race and Sanger (1). The values given were 4+ = 10, 3+

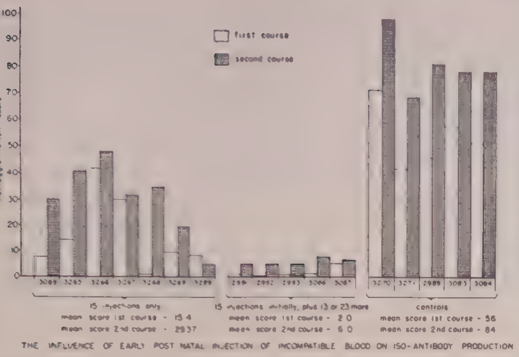

FIG. 1. Influence of early postnatal inj. of incompatible blood on isoantibody production.

= 8, 2+ = 5, 1+ = 2, ± = 1, and 0 = 0. Tiration of each serum against a standard cell was represented by a score, and the scores of the sera from animals in different treatment groups were compared.

*Results.* We were at first concerned with the effect of repeated injection of blood into very young rabbits on their general health and weight gain. Repeated introduction of blood brought no untoward effects; rather treatment appeared to improve the probability of an animal's surviving the critical postnatal period. It is not unusual in breeding of rabbits to suffer the largest loss of animals shortly after birth. There was some postnatal loss in control animals, but we had no loss in animals receiving the equivalent of repeated intraperitoneal transfusions, even though blood was isoantigenically foreign. All animals were bled at regular and frequent intervals from 2 months of age to just before start of our standard immunization procedure. In no case was there evidence of isoantibody production or of normally occurring isoantibody. In our treated animals repeated injections did not stimulate production of anti-A.

After subjection to standard isoimmunization procedure all animals in the non-treated control groups produced uniformly good titres of anti-A; treated animals reacted in a different manner. The treatment groups and schedules are shown in Table I. Fig. 1 illustrates the results graphically. It can be seen that the amount of antibody produced is dependent upon postnatal treatment. Comparison of mean scores, when animals are divided into 3 groups based upon treatments, clearly indicates that there is a marked difference. Mean scores of these groups are statistically highly significantly different from one another.

*Discussion.* In the present study, a single-red-cell incompatibility system was used, and the only known variable was the type of post-

TABLE I. Summary: Treatment to Induce Acquired Tolerance to Incompatible Rabbit Blood in Newborn Rabbits.

| Treatment group | Initial, 15 inj. | No. of inj. after first 15 | Hemagglutination score after standard immunization procedure as young adults | | |
|---|---|---|---|---|---|
| | | | 1st course | 2nd course | |
| a | None | None | 51 | 78+ | Control |
| | + | 23 | 1 | 5 | Inj. 2 times/wk after first 15 |
| | + | 23 | 0 | 5 | |
| | + | 23 | 2 | 5 | |
| b | None | None | 58 | 80+ | Control |
| | ” | ” | 53 | 78+ | ” |
| | + | 13 | 2 | 8 | 1 time/wk after first 15 |
| | + | 13 | 5 | 8 | |
| | + | None | 7 | 30 | |
| c | + | None | 14 | 40 | |
| | + | ” | 42 | 48 | |
| | + | ” | 28 | 31 | |
| | + | ” | 1 | 35 | |
| | + | ” | 9 | 18 | |
| | None | ” | 73 | 100 | Control |
| | ” | ” | 43 | 70 | ” |
| d | + | ” | 7 | 5 | |

natal treatment. Our results clearly indicate that early postnatal introduction of incompatible blood is capable of markedly altering the antibody response. When only the initial 15 injections were given, productivity was decreased but there was still a substantial amount of antibody in all except one animal (3289). The continued stimulation, even with as little as 0.5 cc/week, was capable of depressing antibody production to a point where the amount produced was negligible. The mean scores of 2 and 6 represent mean titres of less than 1:2; in the rabbit these titres are not considered significant of isoantibody production.

The marked inhibition of isoantibody production does not fit into the typical patterns when compared with the phenomena of acquired tolerance and specific inhibition of antibody production. The antigens we have used are found only on the erythrocyte. Extensive trials by us as well as others(18,19) have failed to disclose these antigens in other tissues such as liver or spleen or on sperm cells or leucocytes. The stimulus for inhibition thus can be attributed only to the antigen of the red cell, and the amount of effective isoantigen on this cell is thought to be relatively small. The inhibition which results from our treatment would therefore be difficult to explain on the basis of prolonged retention of relatively large amounts of antigen. Since this antigen has not been found on the leucocytes, an explanation relying on implantation and reproduction of living cells is not acceptable.

It is clear that no matter what mechanism is responsible for our results, we have been able to alter markedly the reaction of a rabbit to isoantigen. Whether a similar phenomenon can occur by prenatal treatment of the fetus is now being investigated.

*Summary*. Introduction of incompatible whole blood into newborn rabbits is capable of altering response of these animals to a later encounter with the same isoantigen. An initial 15 injections given from the day of birth decreased isoantibody production significantly, but not completely. Injections given in addition to the initial 15 effected complete inhibition of specific isoantibody production.

The author gratefully acknowledges the technical assistance of Robert Schuster.

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# Concentrative uptake of 6-Diazo-5-Oxo-L-Norleucine by Sarcoma 180, Liver and Muscle *in vivo*.*† (24436)

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The amino acid analogs O-diazo-acetyl-L-serine (azaserine) and 6-diazo-5-oxo-L-norleucine (DON) are of interest because of their effects in many biological systems and in particular because of their inhibitory effects on tumors(1-7). Both azaserine and DON block *de novo* synthesis of purines by inhibiting an enzyme involved in conversion of α-N-formylglycinamide ribotide to α-N-formylglycinamidine ribotide(8-10). In short term experiments, incorporation of glycine into purines is markedly inhibited by azaserine but there is no inhibition of glycine incorporation into proteins(8,11). Tumor cells and normal cells compete for essential nutrients in the extracellular space. The cell type which can attain the highest intracellular-extracellular concentration gradients of an essential metabolite may be considered to have a competitive advantage. Christensen and his coworkers have evidence that rapidly growing tissues, foetal, regenerating, and neoplastic, concentrate amino acids to a greater extent than do many adult, non-regenerating tissues (12-14). We have shown(15) that azaserine and DON are concentrated intracellularly in Ehrlich ascites cells by the amino acid transport system. It was suggested that perhaps a relatively greater concentration of these compounds intracellularly in tumors, contributes to tumor inhibition obtained in chemotherapeutic tests. The present study was therefore undertaken to measure intracellular-extracellular concentration gradients of DON in liver, skeletal muscle and sarcoma 180 (S 180) *in vivo*.

*Materials and methods.* The S 180, obtained from Dr. D. A. Clarke of Sloan-Kettering Institute, and carried subcutaneously in adult Swiss albino ICR mice obtained from Millerton Farms, Millerton, N. Y. Tumor-bearing mice were used for experiments 6-8 days after subcutaneous implantation of S-180. To determine concentration of DON in the intracellular and extracellular water it was necessary to measure DON and water content of a sample of plasma, and total DON, total water, and the fraction of total water in the extracellular space of a sample of each of the tissues. The procedures used follow: Mice were first subjected to a bilateral nephrectomy using ether anesthesia and the dorsal approach to the kidneys, to prevent any loss of large amounts of DON injected. After the mice had recovered from the anesthesia, DON was injected intravenously via the tail vein. The diluent contained sodium chloride and sodium bicarbonate in the molar proportions 5:1 and was diluted to make the final solution approximately isotonic. Heparin was added to a level of 200 units/ml diluent. The animals were sacrificed by decapitation and blood collected in a chilled centrifuge tube. The mice were then chilled in ice water, blotted dry and dissected. Samples of skeletal muscle always consisted of the gastrocnemius and muscles of back of thigh, all freed of any obvious fat, nerve and tendon. Care was taken to dissect the S 180 samples free of necrotic tissue. The fraction of tissue water in the extracellular space was assumed to be closely approximated by the sucrose space. Tissue sucrose spaces were determined on a group of animals bearing 8-day-old S 180. These were handled the same way as those in the DON experiments except that they were not nephrectomized and received 0.5 ml of 0.3 M sucrose containing 1000 units heparin/ml, intravenously. Sucrose was extracted by homogenizing the tissue samples with one ml of 0.5 N NaOH and 8-10 ml of

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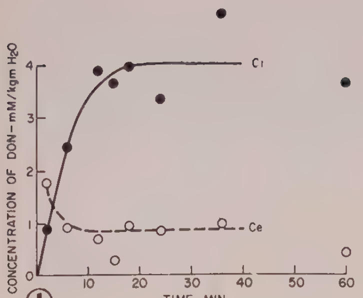

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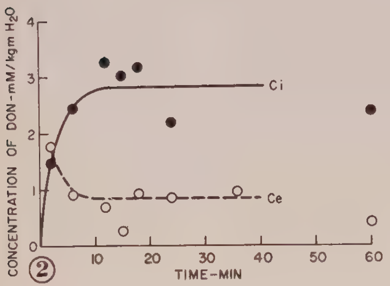

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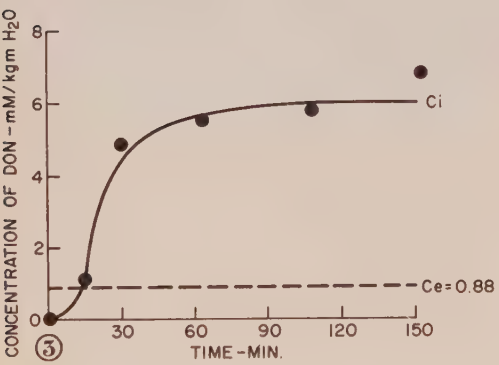

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FIG. 1. Concentrative uptake of DON by S 180 *in vivo*. Ci = intracellular concentration; Ce = extracellular concentration.

FIG. 2. Concentrative uptake of DON by mouse liver *in vivo*.

FIG. 3. Concentrative uptake of DON by slices of S 180 suspended in Krebs-Ringer bicarbonate.

water, adding one ml 10% $Zn\ SO_4 \cdot 7H_2O$, homogenizing and centrifuging. The precipitate was washed once with 10 ml distilled water. The supernatants were combined and diluted to 25 ml. Sucrose was determined by the method of Roe *et al.*(16). Average values for the fraction of tissue water in the extracellular space were, liver—0.16, skeletal muscle—0.14, S 180—0.36. These of course hold only for tissues partly drained of blood by the sampling procedure used. The DON was determined spectrophotometrically on 75% ethanol-barium acetate extracts of tissue homogenates or plasma by method previously described(15). Intracellular concentrations were calculated as though the DON were equally distributed throughout the intracellular water, from the formula,

$$C_i = \frac{A - mh\ We\ Ce}{mh\ (1 - We)}$$

wherein, A is total DON in the tissue sample, m is wet weight of tissue sample, h is water content of the sample in g $H_2O$/g wet wt., We is sucrose space for the tissue and Ce extracellular concentration of DON in mM/kilo of water. Extracellular concentration of DON was assumed equal to concentration in plasma water.

*Results.* Fig. 1 and 2 show the time course of DON uptake in S 180 and liver respectively. Although there was considerable variation from animal to animal, intracellular concentrations in S 180 were consistently higher than in the liver of the same animal once the plateau of the uptake curves was reached. Muscle gave no evidence of concentrative uptake, intracellular concentrations being 0.5-1.5 times extracellular.

One experiment was made with S 180 slices incubated in Krebs-Ringer-bicarbonate containing DON, to see if higher intracellular concentrations could be obtained, since it would be expected that competition from normal free amino acids would give somewhat lower values for DON concentration intracellularly *in vivo*. To check this an extracellular concentration close to that obtained in the experiment shown in Figs. 1 and 2, was chosen. The time course of DON uptake is shown in Fig. 3. Intracellular concentration attained *in vitro* was 1.5 times as high as *in vivo*. As expected, the time to reach the steady state in the S 180 slices is much longer than *in vivo*. This is no doubt due to the much slower diffusion into the extracellular space in slices *in vitro*.

*Discussion.* The present findings in conjunction with previous results on concentrative uptake of DON by Ehrlich ascites cells (15) and data on concentrative uptake in normal tissues(17) readily explain the findings(4) that DON rapidly disappears from blood after intravenous injection and that only minute amounts of it are excreted in the urine. In common with the normal amino acids it would be reabsorbed in the renal tubules and be concentrated intracellularly by the amino acid transport system in many of the visceral tissues. Unfortunately relative concentrations of DON in viscera other than liver were not obtained. Studies on duodenum and kidney would be of particular interest since Noall *et al.*(17) have shown that α-aminoisobutyric acid is concentrated to a greater extent in duodenum and kidney than in liver. It is likely that the general pattern of concentration intracellularly will follow that of the normal amino acids.

The high toxicity of DON is probably explained by inhibition of purine synthesis(8-11) plus its concentration intracellularly by the amino acid transport system. This in itself does not explain the selective inhibition of many transplantable tumors. A somewhat greater concentrative uptake of DON by tumors in conjunction with a relatively greater importance of the *de novo* pathway of purine synthesis in tumors(18,19) may account for the inhibitory effect on transplantable tumors.

Ackerman and Potter(20) have proposed that inhibition of an enzyme which is present in lower concentrations in a tumor than in normal tissue may provide a theoretical basis for cancer chemotherapy. However, the presence of a lower concentration of an enzyme in a tumor may imply that the metabolic pathways in which this enzyme is involved are of less importance in metabolism of the tumor than of normal tissues. We suggest the following alternative generalizations. Active chemotherapeutic compounds are more likely to be compounds which, a) are actively concentrated by tumor cells to a higher intracellular concentration than by normal cells, or b) inhibit enzymes of metabolic pathways which are more active in tumor cells than in normals. Useful compounds are more likely to be found among those which satisfy both of these conditions.

*Summary.* DON is concentrated intracellularly in S 180 and liver to levels 5-fold and 4-fold higher respectively than the extracellular concentration at a plasma level of 0.8-0.9 mM/kilo of plasma water. Intracellular concentrations in skeletal muscle were the same or less than the extracellular.

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# Biophysical Characterization of Infectious Bovine Rhinotracheitis Virus. (24437)

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The agent of infectious bovine rhinotracheitis (IBR), an acute upper respiratory disease of cattle, has been isolated from nasal washings of infected cattle on bovine kidney tissue culture. The viral nature of the etiologic agent has recently been established by extensive serologic and transmission studies(1-5). The virus is cytopathogenic to cells of both bovine kidney and human amnion cultures in which it induces development of intranuclear inclusions, readily observed in stained preparations(6). The agent is relatively stable and can be held at 4°C over 30 days and at 22° C for 3 days with no measurable loss of titer(7). This study was undertaken to demonstrate the entity associated with the infectious unit and to determine its physical characteristics.

*Materials and methods. Tissue culture.* Bovine kidney cell cultures were prepared by the method of Madin *et al.*(8) with a medium of 0.5% lactalbumin hydrolysate in Hanks balanced salt solution containing .01 M Tris buffer, with addition of 5% lamb serum. Human amnion cell cultures were prepared by the method of Zitcer *et al.*(9) with a medium of bovine amniotic fluid containing 5% horse serum. *Virus.* Pritchard and American strains of IBR virus as described by Cheatham and Crandell(6) were used. Virus pools of Pritchard strain were prepared by inoculating the agent in bovine kidney cultures into 32 oz. prescription bottles. When the cytopathogenic effect was complete at about 72 hours, the fluid was frozen and thawed several times to disrupt the infected cells, then centrifuged in refrigerated centrifuge at 2,000 rpm for 10-15 minutes to remove cellular debris. The supernate was centrifuged at 20,000 rpm in No. 21 rotor of Spinco model "L" preparative centrifuge for one hour; the sediment was resuspended in phosphate buffered saline of pH 7.4 and again centrifuged at 5,000 rpm for 15 minutes in the same rotor. The infectious fluid was dispensed into glass ampules, sealed and stored at –60°C. Ten-fold serial dilutions of the virus pool, $10^{-1}$ through $10^{-7}$, were made in tissue culture medium. One-tenth ml volumes of each dilution were inoculated into each of 4 bovine kidney tissue culture tubes. The tubes were observed daily 5 days until a cytopathogenic end point was reached. The virus pool titered $10^{-6.5}$/0.1 ml throughout experimental period. Tissue culture infective dose 50% ($TCID_{50}$) was calculated by the method of Reed and Muench(11). Both analytical and preparative Spinco centrifuges were used for identification of infectious particle. Since concentration of the infectious agent was not high enough to permit optical detection of the sedimenting boundary in the analytical centrifuge cell, pseudovelocity procedures were employed using methods described previously(13) for identification of the virus particle. Experiments in the preparative Model "L" centrifuge were carried out using the SW-39 rotor. Centrifugation speeds were 5,000, 10,000, 15,000, 20,000, and 25,000 rpm for each of 10, 15, 20 and 25 minute periods. At the end of each run, samples were withdrawn from each third of tube (top, middle and bottom), and titrated. Using standard centrifugation relations(12), sedimentation coefficients‡ were computed for each level of tube at indicated velocities. A 2-log unit loss in infectivity was interpreted

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[‡] The sedimentation coefficient $S = \left(\frac{1}{\omega^2}\right) \cdot \left(\frac{1}{t_2 - t_1}\right) \cdot \left(\frac{x_2 - x_1}{x}\right)$, where $\omega$ is the centrifugal field, $(t_2 - t_1)$ is time necessary for 2-log unit drop of infectivity in radial distance $(x_2 - x_1)$ of rotor with radius x.

TABLE I. Particle Size of IBR Virus as Determined by Ultracentrifugation.

| Exp. No. | Method | Calculated virus particle diameter<br>mμ | | mμ |
|---|---|---|---|---|
| 2 E | Pseudo-velocity | >125 | | |
| 3 E | | >146 | & | <166 |
| 6 E | | >105 to 137 | | ≧152 & <160 |
| 1 L | SW-39 rotor | <210 | | |
| 2 L | | > 95 | & | ≧148 |
| 3 L | | >121 | & | ≧151 |
| 4 L | | ≧150 | & | <210 |

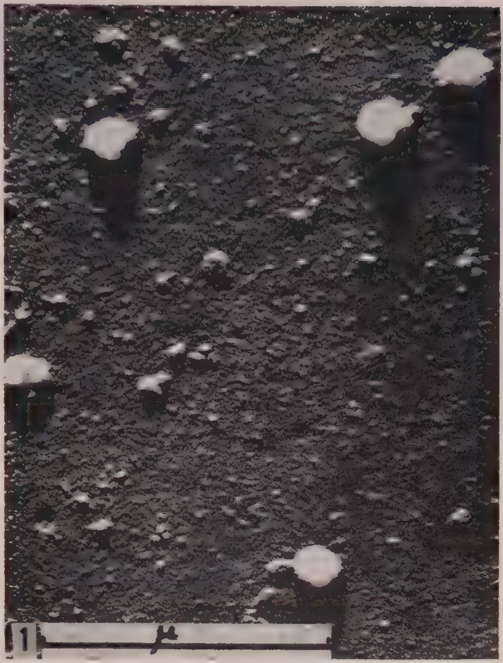

FIG. 1. Electron micrograph of IBR virus prepared by agar pseudoreplica method directly from stock solutions used in ultracentrifugation experiments without further concentration or fixation. Shadowed with uranium at arc tan 1/3. × 38,400.

as time necessary for virus particle to travel the distance ($x_2 - x_1$) used in these calculations. Spherical particle diameters were computed from corrected sedimentation coefficient to water and 20°C ($S_{20,w}$) of the cytopathogenic agent, the viscosity of medium ($\eta$) and the difference between particle and medium densities.§ Specimens for electron microscopy studies were prepared from stock virus by various methods, such as air drying, agar pseudoreplica(17) or fixed in formaldehyde and dialyzed against distilled water before air drying. They were shadowed with uranium at an angle of arc tan ⅓ and examined with a RCA electron microscope EMU-3B. At intervals during cytopathogenic period, human amnion cultures infected with American strain of IBR were fixed 2 hours with 1% osmium tetroxide in isotonic veronal buffer of pH 7.35 and scraped from wall of tubes. The infected cells were dehydrated in graded ethanol and embedded in methacrylate by the method of Tousimis and Hilleman(10). Ultrathin sections made with a Porter-Blum type ultramicrotome(14) were placed on grids coated with Parlodion or Formvar film and examined in electron microscope without removing the methacrylate.

*Results. Centrifugation.* The results indicated that the particle associated with infectivity has a spherical diameter smaller than 175-210 mμ but equal to or larger than 148-151 mμ. Calculations based on pseudo-velocity experiments carried out with partition cell(15) in the analytical ultracentrifuge, showed that the infectious particle has a hydrated diameter in the same range as that found with the SW-39 rotor (Table I).

Examination with the electron microscope of suspensions of the IBR agent in buffered isotonic saline, clarified from infected bovine kidney tissue culture cells, revealed particles as seen in Fig. 1. The majority of particles in these suspensions were 153 ± 12 mμ in width when prepared by agar pseudoreplica method and shadowed with uranium. On the average about 20% flattening of these particles was measured from shadowlengths. Polysterene latex spheres of 260 mμ in diameter were used to determine local shadow angles(16). Assuming that the virus has flattened to an oblate spheroid upon drying(10), its calculated diameter is 136 mμ with 10.8 mμ % standard deviation. Particles of this diameter were not found in normal control preparations.

In virus suspensions fixed in 2% formaldehyde before preparation for electron microscopy, particles with very little flattening and widths of 137 ± 9 mμ were observed. Many of these particles had a less electron-dense central core, 35 to 46 mμ in width, and others were irregular in shape and contained round dense masses of about 50 mμ (Fig. 2A and 2B).

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§ Particle diameter (cm) $d = \sqrt{\dfrac{18\,\eta\, S_{20,w}}{\rho_2 - \rho_1}}$, where ($\eta$) is viscosity of medium, $S_{20,w}$ is sedimentation coefficient and ($\rho_2 - \rho_1$) density difference between particle and medium.

In addition to the 136 mμ diameter particle, we found smaller particles of about 110 mμ in width and others of 40 mμ or less. The smaller particles (40 mμ or less) were eliminated by differential centrifugation without any detectable change in cytopathogenicity of the preparation. Particles of the 110 mμ size were also found in normal control cell homogenates. This observation is not sufficient to eliminate any possible association of these particles with the virus itself. The possibility that these are the incomplete or dissociated virus can not be excluded. The presence of high numbers of particles smaller (40 mμ or less) than the virus in infected cultures suggests that these are cellular components or the product of cell degeneration. Correlation of the number of virus particles as measured in electron micrographs with cytopathogenic units(13), showed that approximately 2 x $10^9$ particles/ml correspond to infectivities of $10^7$ cytopathogenic units/ml.

Electron microscopy of ultrathin sections of human amnion tissue culture cells infected with IBR virus showed numerous intranuclear particles of 125 to 140 mμ in width (Fig. 3). At later stages of infection (12 days) these virus-like particles were found in the nucleus and cytoplasm and also attached to the surface of cells (Fig. 4).

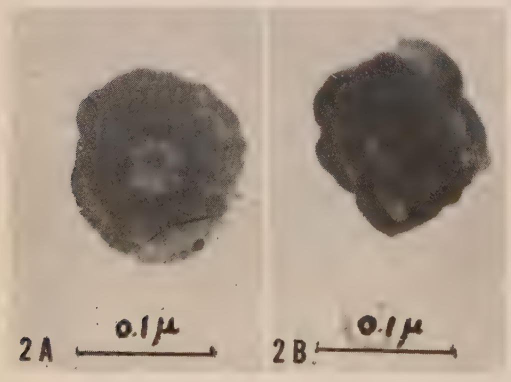

FIG. 2A and 2B. Formaldehyde-fixed virus particles for 20 min., dialyzed against distilled water for 24 hr and air dried on parlodion-coated grids. Shadowed lightly with uranium. × 180,000.

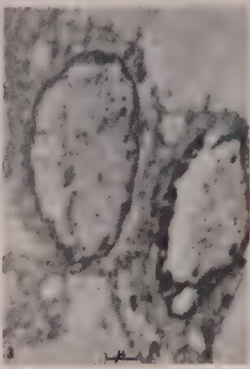

FIG. 3. Human amnion tissue culture cells 10 days after infection with IBR. Virus-like particles are seen within the nucleus. $O_sO_4$ stain and methacrylate embedded. × 4,600.

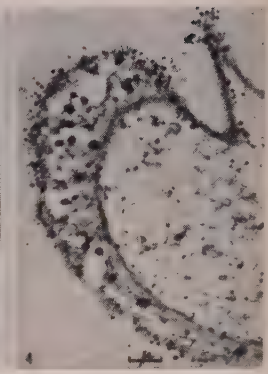

FIG. 4. Portion of a human amnion cell 12 days after infection. Virus-like particles appear in the enlarged nucleus and on cell surface. × 4,600.

*Summary.* The size of infectious bovine rhinotracheitis virus grown in bovine kidney tissue culture, based on ultracentrifugation results is between 145-156 mμ in diameter. Electron microscopical examination of infectious fluids revealed particles of 136 ± 10.8 mμ in diameter. Similar particles were observed intracellularly in ultrathin sections of the infected human amnion cells in tissue cultures.

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# Comparison of Monoamine Oxidase Inhibitory Effects of Iproniazid and Its Phenyl Congener.* (24438)

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Iproniazid (1-isonicotinyl-2-isopropylhydrazine) has gained widespread interest in studies of the metabolism of biological amines. Its ability to inhibit monoamine oxidase (MAO) has greatly facilitated the understanding of the properties of this enzyme as well as of pharmacological and biochemical effects of serotonin (5-hydroxytryptamine) and norepinephrine in the brain. The MAO inhibitory properties of iproniazid *in vivo* were earlier described by Zeller *et al.*(1) and Schayer(2). Iproniazid has also been found to be effective in treatment of depressed mental patients. Its use in these cases presumably depends upon inhibition of MAO and prevention of the degradation of endogenous amines of the brain. These and other findings have stimulated the search for newer and more potent inhibitors of MAO. Recently the compound beta-phenylisopropylhydrazine[†] (PIH) was found to be some 30-50 times as potent as iproniazid in inhibiting MAO both *in vitro* and *in vivo*(3). The present report describes the effect of a closely related compound, 1-isonicotinyl-2-phenylisopropylhydrazine dihydrochloride[‡] (isonicotinyl-PIH) on MAO activity. Isonicotinyl-PIH also resembles iproniazid, differing only by the presence of a phenyl group on the isopropyl chain. The similarities in structure of the 3 compounds are seen in Fig. 1.

*Methods.* Thirty-six male Wistar rats were used. *In vitro* MAO activity was determined by the method described by Sjoerdsma *et al.* (4) and by Bogdanski *et al.*(5). One milliliter of liver homogenate, 200 mg/ml was incubated with 4 $\mu$M of serotonin creatinine sulfate at pH 7.0. The inhibitors were added to the enzyme preparations and incubated 15 minutes prior to addition of substrate. Total volume of the incubation mixture was 3 ml. All incubations were made at 38°C in a constant temperature shaker. After incubation for 30 minutes the mixtures were extracted and assayed for residual serotonin according to the nitrosonaphthol method of Udenfriend, *et al.*(6). Readings were made on a Beckman DU spectrophotometer. *In vitro* studies were carried out using concentrations up to $10^{-4}$ M of the inhibitors. The effect of iproniazid and isonicotinyl-PIH on liver and brain MAO *in vivo* was also investigated. A dose of $5 \times 10^{-5}$ M/kg of inhibitor was injected subcutaneously 2 hours prior to sacrifice of the animals. Tissues were quickly removed and homogenized and diluted to a concentration of 20% (liver) and 33% (brain). One

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[*] This work was supported by grants from Lakeside Laboratories, Milwaukee, Wis., and Fund 171, State of Washington.

[†] Beta-phenylisopropylhydrazine is designated as Compound JB-516, Lakeside Labs.

[‡] The compound 1-isonicotinyl-2-phenylisopropylhydrazine dihydrochloride (isonicotinyl-PIH) was furnished for these studies by Lakeside Labs., under the designation of JB-821. Iproniazid was obtained through the courtesy of Dr. R. S. Floody, Hoffmann-LaRoche, Nutley, N. J.

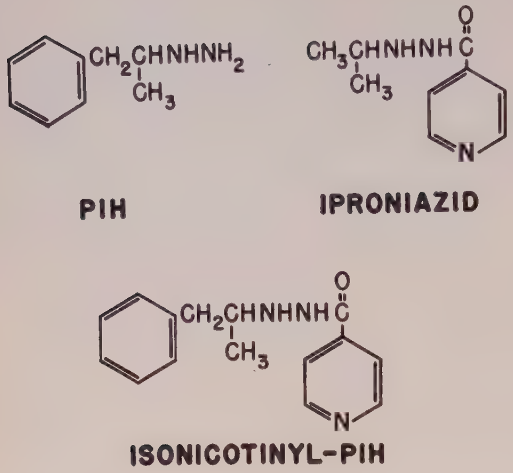

FIG. 1.

milliliter of the homogenate was incubated with the serotonin as described in the *in vitro* studies. Incubations with brains were carried out for 1 hour. The degree of MAO inhibition was calculated on the ability of the drug to decrease the rate of serotonin breakdown under the conditions employed. Results are graphed as % serotonin metabolized by the homogenates in the absence and presence of inhibitor.

*Results.* The results of *in vitro* inhibition of liver MAO by iproniazid and isonicotinyl-PIH are summarized in Fig. 2. Control homogenates metabolized approximately 70% of serotonin present during 30 minutes of incubation. In the presence of $10^{-4}$ M inhibitor, both iproniazid and isonicotinyl-PIH showed complete inhibition. At $5 \times 10^{-5}$ M, inhibition by isonicotinyl-PIH was still essentially complete while the iproniazid effect had weakened and permitted about a 35% breakdown of substrate. When the concentrations of inhibitors were decreased to $10^{-5}$ M, serotonin breakdown occurred in both cases, but some inhibition was still present. Inhibition produced by iproniazid at this concentration was approximately 20% while isonicotinyl-PIH still exerted a 40% block. At both $5 \times 10^{-5}$ and $10^{-5}$ M the difference in degree of MAO inhibition exerted by the 2 drugs was highly significant ($P<0.01$).

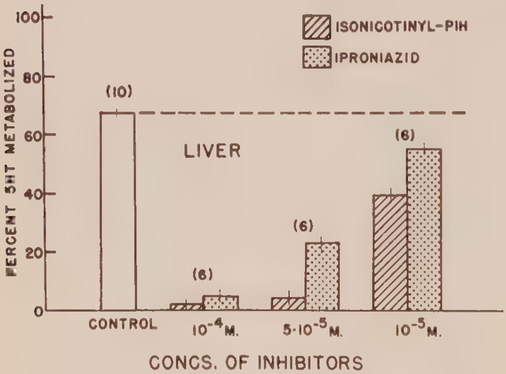

FIG. 2. Graph representing mean values of percent serotonin metabolized by rat liver homogenate in absence and presence of various concentrations of inhibitors. Vertical lines represent stand. error of mean values. Figures in parentheses indicate No. of experiments in each group.

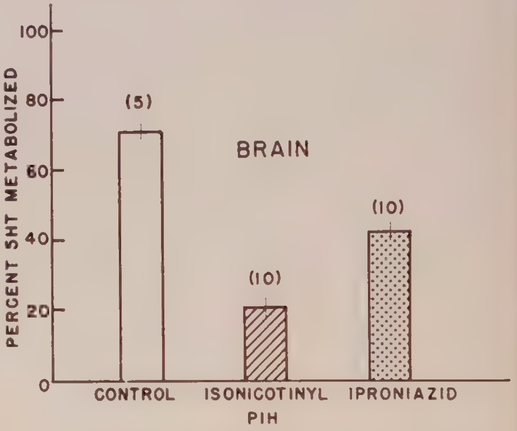

FIG. 3. Graph representing mean values of percent serotonin metabolism by brain homogenates prepared from control rats and rats pretreated with $5 \times 10^{-5}$M/kg of iproniazid and of isonicotinyl-PIH. Vertical lines represent stand. error of means. Figures in parentheses indicate No. of animals used in each group.

Liver and brain homogenates prepared from rats pretreated with the inhibitors showed results similar to the *in vitro* studies. At doses of 2-3 $\times 10^{-5}$ M/kg, liver MAO was completely inactivated whereas the brain enzyme was still active. The dose of $5 \times 10^{-5}$ M/kg was selected since both inhibitors produced sufficient inhibition to allow comparison of their relative effectiveness against brain MAO. Fig. 3 indicates activities of brain homogenates from control and inhibitor-treated rats in metabolizing serotonin. Isonicotinyl-PIH is approximately twice as effective as iproniazid in these preparations, showing a 70% inhibition. As in the *in vitro* results the difference in degree of inhibition by the 2 drugs was highly significant ($P<0.001$).

*Discussion.* The results of these experiments indicate that addition of a phenyl grouping to the isopropyl chain in iproniazid results in a compound with greater MAO inhibiting properties. Comparing the results obtained earlier with beta-phenylisopropylhydrazine (PIH)(3) it is also evident that the addition of an isonicotinyl group to the terminal N greatly decreases this property. This agrees with the studies of Davison(7) in which isopropylhydrazine was shown to be a much more potent MAO inhibitor than its isonicotinyl derivative (iproniazid). Although the effectiveness of MAO inhibition is reduced by addition of the isonicotinyl group to PIH, some of the pharmacological effects of the latter compound are also reduced or eliminated (unpublished observations). Studies of these compounds thus far indicate that isonicotinyl-PIH resembles iproniazid in its MAO inhibitory actions as well as in its pharmacological properties more closely than PIH. Such factors must be taken into consideration in the event that these compounds become useful therapeutic agents.

*Summary.* Comparison of monoamine oxidase (MAO) inhibiting properties of iproniazid and its phenyl congener, 1-isonicotinyl-2-phenylisopropylhydrazine (isonicotinyl-PIH) showed it to be 1.5 to 2 times as effective in inhibiting MAO both *in vitro* and *in vivo*. The results indicate that the addition of a phenyl group to the isopropyl chain of iproniazid enhances MAO inhibitory properties. Comparisons with beta-phenyl-isopropylhydrazine also indicate that the isonicotinyl group hinders the MAO inhibiting property of the former compound.

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# Antitumor Activity of 5-Bis(2-Chloroethyl) Amino Uracil Against Sarcoma 180 and Ehrlich Carcinoma. (24439)

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Incorporation of chloroethylamine groups into compounds of biological activity may lead to compounds which have selective action on certain types of neoplasms. l-Phenylalanine nitrogen mustard (CB-3025)(1), quinacrine nitrogen mustard(2), and Degranol (BCM)(3) may be considered as examples of compounds of this type. Lyttle and Petering (4) have reported synthesis of 5-bis(2-chloroethyl) amino uracil (U-8344). Antitumor activity of the compound was demonstrated against 2 rat tumors, Walker adenocarcinoma 256 and the Murphy-Sturm lymphosarcoma. This report describes antitumor activity of U-8344 in 2 mouse tumors, Sarcoma 180 and Ehrlich carcinoma. Chlorambucil (CB-1348) was included in the tests as a reference compound.

*Experimental.* The acute $LD_{50}$ in Swiss mice for U-8344 and chlorambucil (CB-1348) was 3.5 mg/kg and 63.5 mg/kg, respectively, when the drugs were given in a single intraperitoneal injection and animals observed for 7 days. U-8344 was screened against the ascitic form of Ehrlich carcinoma.† Groups of 10 female Swiss mice were implanted intraperitoneally with 4.2 million tumor cells. Treatment was started 16-20 hours later. The drug was dissolved in 95% ethyl alcohol and stored at 4°C. Solutions were prepared daily for injection by diluting aliquots with 1% carboxymethyl cellulose. Injections were

TABLE I. Effect of U-8344 against Ehrlich Carcinoma (Ascites).

| | | 8th day | | 13th day | |
|---|---|---|---|---|---|
| | Dosage (mg/kg/day) | Change in body wt (g) | Deaths | Change in body wt (g) | Deaths |
| Tumor control | | + 8 | 0 | + 14 | 1 |
| Non-tumor control | | + 2 | 0 | + 3 | 0 |
| U-8344 | .8 | + 4 | 0 | + 9 | 0 |
| " | .4 | + 4 | 0 | + 8 | 2 |
| " | .2 | + 5 | 0 | + 12 | 1 |
| " | .1 | + 9 | 0 | + 16 | 3 |

TABLE II. Effect of U-8344 and Chlorambucil on Survival of Mice with Ehrlich Ascites.

| Preparation | Dosage (mg/kg/day) | Median days | Survival time (stand. error) | P* | Alive at 82 days |
|---|---|---|---|---|---|
| U-8344 | .2 | 19 | (4.0) | .50 | 2 |
| | .4 | 34 | (3.8) | <.01 | 5 |
| | .8 | 43 | (7.9) | <.01 | 5 |
| | 1.2 | 30 | (6.1) | .02 | 2 |
| | 1.6 | 27.5 | (4.7) | .02 | 2 |
| Chlorambucil | 5.0 | 16.5 | (1.3) | .76 | 0 |
| | 10.0 | 19.0 | (1.4) | .07 | 0 |
| | 20.0 | 12.5 | (2.2) | .13 | 0 |
| Tumor control | | 16.0 | (0.9) | | 0 |
| Non-tumor control | | | | | 16 |

* P = Approximate probability of observed deviation from control median ST (normal deviate test).

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* The authors wish to acknowledge contribution of Dr. O. S. Carpenter who made statistical analysis of the data.

TABLE III. Effect of U-8344 and Chlorambucil on TPCV of Ascitic Tumor.

| | | Ehrlich ascites | | | | | | Sarcoma 180 ascites | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | TPCV, ml | | | | | | TPCV, ml | |
| Preparation | Dose, mg/kg/day | No. survivors | No. no cells | P* | Avg fluid vol, ml | Avg | S.E. | No. survivors | No. no cells | P* | Avg fluid vol, ml | Avg | S.E. |
| Tumor control | | 36 | 1 | | 7.59 | 2.01 | .13 | 30 | 1 | | 6.18 | 1.65 | .09 |
| Chlorambucil | 5 | 12 | 0 | .56 | 3.29 | 1.05 | .12 | 14 | 0 | .69 | 3.93 | 1.04 | .07 |
| | 10 | 15 | 13 | <.01 | .35 | .08 | .07 | 15 | 6 | <.01 | 1.10 | .36 | .09 |
| | 20 | 15 | 14 | " | .33 | .09 | .09 | 15 | 14 | " | .77 | .03 | .03 |
| U-8344 | .1 | 15 | 2 | .42 | 4.61 | 1.07 | .14 | 15 | 0 | .73 | 5.35 | 1.26 | .08 |
| | .33 | 15 | 14 | <.01 | .23 | .06 | .06 | 15 | 15 | <.01 | .0 | <.01 | |
| | 1 | 13 | 13 | " | .0 | <.01 | | 14 | 14 | " | .0 | " | |

* P = Probability of chance deviation from control in No. with no cells as large as observed deviation (Chi-square test).

made subcutaneously twice daily for 7 days. Mice were weighed at the start of the experiment and on $T_8$[‡] and $T_{13}$. U-8344 in doses of 0.4 to 0.8 mg/kg/day produced a 50% inhibition ($T_8$) of body weight gain of ascitic mice (Table I). Inhibition of development of ascitic fluid is noted also on the 13th day.

U-8344 was also tested against the Ehrlich carcinoma using survival as the index of activity. Chlorambucil was used as a standard drug for comparative purposes. Solutions of U-8344 and chlorambucil were made in ethyl alcohol. Dilutions were made daily with saline. The drugs were injected intraperitoneally once daily for 3 days into groups of 20 female mice which had been implanted with 4.0-4.5 million tumor cells. Survivors were checked daily. The experiment was terminated on the 82nd day. Average survival time was calculated by assuming that survivors died 24 hours after termination date. Approximately 30% of survivors in all groups showed solid tumors at site of injection. U-8344 was more effective than chlorambucil in prolonging survival time of mice with Ehrlich carcinoma in the doses used. (Table II).

U-8344 was tested against the ascitic forms of Ehrlich carcinoma and Sarcoma 180, using the total packed tumor cell volume (TPCV) technic. The drugs were dissolved in ethyl alcohol and diluted daily with saline. Injections were made once daily for 7 days, beginning 20 hours after implanting the tumor. Total packed tumor cell volume was determined 24 hours after last injection. Fifteen mice were used per dosage level; 40 mice were used for Ehrlich and 30 were used for Sarcoma 180 ascites controls. U-8344 is effective against the 2 ascitic tumors and is more effective than chlorambucil on either a weight or molar basis (Table III).

One-tenth ml of a homogenate of Sarcoma 180 was implanted subcutaneously in

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[†] The Ehrlich carcinoma (ascites form) was obtained from Dr. T. S. Haushka, of Roswell Park Memorial Inst. and has been carried in our laboratory since May 1956. Sarcoma 180 was obtained from Dr. K. Sugiura, of Sloan-Kettering Memorial Inst. and has been carried in our laboratory since Jan. 1957.

[‡] $T_8$ refers to 8th day after transplanting tumor.

TABLE IV. Effect of U-8344 against Sarcoma 180.

| Preparation | Dosage (mg/kg/day) | Change in body wt (g) | Tumor wt mg | (S.E.) | P* | % tumor inhibition | Deaths |
|---|---|---|---|---|---|---|---|
| U-8344 | .2 | + 1 | 474 | ( 52.3) | .32 | 13 | 0 |
| | .4 | 0 | 281 | ( 48.2) | .05 | 49 | 0 |
| | .8 | − 3 | 94 | ( 16.1) | <.01 | 83 | 0 |
| Tumor control | | + 3 | 546 | (142.2) | | | 0 |
| U-8344 | .8 | − 5 | 67 | ( 11.9) | .01 | 69 | 0 |
| | 1.2 | − 4 | 29 | ( 7.2) | <.01 | 87 | 2 |
| | 1.6 | −10 | 15 | ( 2.4) | <.01 | 93 | 7 |
| Tumor control | | 0 | 218 | ( 54.6) | | | 0 |

* P = Probability of observed deviation from control avg (t-test).

TABLE V. Effect of Dosage Schedule on Inhibition of Sarcoma 180 by U-8344.

| Preparation | Dosage* (mg/kg/day) | Dosage schedule | Change in body wt (g) | Tumor wt mg | (S.E.) | P† | % tumor inhibition | Deaths |
|---|---|---|---|---|---|---|---|---|
| U-8344 | .8 | $T_1$, $T_4$, $T_7$‡ | + 1 | 338 | ( 54.2) | .05 | 41 | 1 |
| | 1.2 | ” | − 1 | 200 | ( 21.4) | <.01 | 65 | 1 |
| | 1.6 | ” | − 7 | 172 | ( 41.6) | ” | 70 | 1 |
| Tumor control | | | + 3 | 571 | (123 ) | | | 0 |
| U-8344 | .8 | $T_5$, $T_8$, $T_{11}$‡ | 0 | 770 | (105.3) | .03 | 36 | 0 |
| | 1.2 | ” | − 4 | 743 | (161.0) | .04 | 38 | 0 |
| | 1.6 | ” | − 8 | 717 | ( 69.1) | .02 | 40 | 2 |
| Tumor control | | | − 2 | 1200 | (171.9) | | | 2 |
| U-8344 | .8 | $T_1$-$T_7$§ | − 4 | 128 | ( 14.5) | <.01 | 76 | 0 |
| | 1.2 | ” | − 5 | 79 | ( 8.2) | ” | 85 | 1 |
| | 1.6 | ” | − 7 | 37 | ( 7.6) | ” | 92 | 1 |
| Tumor control | | | 0 | 596 | ( 54.9) | | | 0 |

* Drug was given intraper. once daily, diluted in 0.9% NaCl.
† Probability of observed deviation from control avg (t-test).
‡ Sacrificed 4 days after last inj.
§ ” 24 hr ” ” ”.

the groin of Swiss mice. Treatment was started 16-20 hours after tumor implantation and continued daily for 7 days. The tumors were dissected out and weighed on a Roller-Smith balance 16-20 hours after last injection. Drugs were given intraperitoneally in single daily doses in saline. Ten mice were used on each dosage level.

A significant inhibition of tumor weight was observed when 0.8 mg of U-8344 was given per kg of body weight (Table IV).

A preliminary study was made to determine if U-8344 was effective on established Sarcoma 180 tumors. It was found (Table V) that U-8344 was less effective on 5-day-old Sarcoma 180 tumors using dosage schedules that produced significant inhibition of 1-day tumors. Drug levels used produced severe body weight loss. Inanition of the animals may be responsible for a significant portion of the tumor inhibition.

*Conclusion.* 5-Bis(2-chloroethyl) amino uracil (U-8344) is an effective agent against the solid and ascites forms of Ehrlich carcinoma and Sarcoma 180 under specified conditions.

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## Properties of Seizures Induced by Histamine.* (24440)

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Presence of histamine in the central nervous system has been demonstrated(1) but its functional significance is not understood. In previous studies, effects of histamine on the brain have been examined in laboratory animals and responses have varied with method and site of injection. When the drug was administered through a cannula implanted in the lateral ventricles, Feldberg and Sherwood (2) observed profound muscular weakness and signs of autonomic disturbance in the unanesthetized cat. By direct intracerebral injection of histamine in the mouse, however, Haley(3) has referred to the occurrence of clonic convulsions in addition to other toxic manifestations. That histamine may be involved in the seizure mechanism is suggested by some central actions of antihistaminics and the efficacy of these agents in the control of certain seizures. Diphenhydramine is effective in treatment of petit mal epilepsy; it will eliminate from the electrocorticogram seizure discharges experimentally induced in animals (4). Central sympathetic stimulation and a pressor response obtained by injection of histamine into the lateral ventricle of the cat were abolished by previous injection of an antihistaminic in this site(5). Further, a remarkably high incidence of allergy has been observed in children with autonomic(6) and febrile seizures(7) and attention has been drawn to a possible relation between allergy and occurrence of abnormal electroencephalographic patterns(8). The present laboratory investigation was designed to elucidate further the relation of histamine to seizures. Properties of histamine-induced seizures and their modification by various classes of drugs have been studied in both mice and guinea pigs, since these animal species differ markedly in their sensitivity(9,10).

*Methods.* Swiss albino mice weighing 18-22 g, and young guinea pigs, 100-200 g, were used as experimental animals. The intracerebral dose of histamine acid phosphate required to produce convulsions in 97% of animals was established ($CD_{97}$) and the pattern and duration of the seizure examined. Histamine, administered by the method of Haley and McCormick(11), was injected to a depth of 2-3 mm at a point 2 mm from the midline of the skull at the level of the anterior base of the ears. All doses are stated in terms of the salt. A syringe graduated to 0.001 ml and ⅜″, 30 gauge needle facilitated accurate measurement and administration of small quantities of drug; occurrence of leakage along the needle track, as pointed out by Bedford(12), was reduced to a minimum. Possible reactions due to penetration and trauma of the brain by the hypodermic needle or volume of fluid *per se* were investigated. Effects of distilled water and isotonic saline were compared with those of phosphate buffer solutions of various pH values. Since histamine acid phosphate in isotonic saline was markedly acidic, the drug was administered in phosphate buffer solution at pH 5.25. Anticonvulsants, tranquilizing agents, antihistaminics and adrenocortical steroids were tested for ability to abolish or modify the pattern of seizures induced by the $CD_{97}$ of histamine. In mice, except for antihistaminics administered by the intraperitoneal route in one experiment and adrenocortical steroids which were injected subcutaneously, all drugs were given orally and dissolved or suspended in 10% acacia solution, of approximate volume 0.4 ml per 20 g body weight. In guinea pigs, drugs were administered by the intraperitoneal route, with the exception of serpasil which was given orally; the fluid volume injected was approximately 0.5 ml per 100 g body weight. Five animals were tested at

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† During tenure of Riker Fellowship awarded by International Council of Pharmacol.

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each dose level and responses compared with a control group given histamine alone. Apart from adrenocortical steroids, drugs were examined for time of peak activity. For determination of anticonvulsant potency, groups of 5 mice given graded amounts of drug were challenged with the $CD_{97}$ of histamine at the time of peak effect, until points were established between complete and no protection. Results were plotted on logarithmic probability paper and a regression line drawn. From these data, the $ED_{50}$ was determined and 95% confidence limits calculated. The anticonvulsant $ED_{97}$ in mice was used for tests of anticonvulsant drug action in guinea pigs. Anticonvulsant and antilethal effects of tripelennamine, diphenhydramine and chlorpromazine were compared in guinea pigs and mice; 5 animals were challenged with an intracerebral $CD_{97}$ of histamine at one half hour after intraperitoneal administration of a 25 mg/kg dose of each antihistaminic. In addition to observations of the convulsant action of histamine when injected intracerebrally, effects of intraperitoneal injection were investigated in mice subjected to electroshock and pentylenetetrazol (metrazol) seizures. The threshold to maximal electroshock seizures in m.amps. was determined 45 min. before and 5 min., 1 and 2 hr subsequent to injection of histamine in doses of 5-2000 mg/kg. A group of control mice was tested at the same time intervals. Similar doses of histamine were administered to mice given a $CD_{97}$ of metrazol (85 mg/kg) by subcutaneous injection after an interval of 5 min. Time of onset and severity of the seizure were compared in control animals not treated with histamine.

*Results. Induction of seizures by intracerebral histamine.* A momentary decrease in motor activity followed penetration of the brain with the needle and injection of a volume of distilled water or isotonic saline, 0.01 ml in the mouse and 0.02 ml in the guinea pig; convulsions did not result. No untoward reactions occurred in animals injected intracerebrally with solutions of M/15 $KH_2Po_4$ and $Na_2HPo_4$ at pH values from 4.52 to 8.83. Histamine acid phosphate, 200-500 $\gamma$ in 0.01 ml of isotonic saline, had a pH value of 3.9. When dissolved in a phosphate buffer of pH 8.2, the pH of histamine solution was 5.25. Histamine, 200-500 $\gamma$ (10-25 mg/kg) by intracerebral injection in the mouse, produced the following seizure pattern: onset within 3-5 min. with circling and rolling toward the side of injection; hyperkinetic behavior and tremor were followed by intermittent clonic jerks and flexor and extensor spasms of fore- and hind-limbs; severe generalized clonus, the principal component of the seizure, was repeated at irregular and short intervals and was succeeded by tonic extension of all limbs and death of the animal within 5-30 min. Occasionally, tonic extension of hind-limbs occurred at the onset of the seizure. The $CD_{97}$ of intracerebral histamine in the mouse was 25 mg/kg; at doses below 25 mg/kg but, greater than 10 mg/kg, occurrence of convulsions was less constant. Injection of 0.25-10 mg/kg of histamine caused tachypnea, defecation, urination, vasodilation and irritation of the ears and occasionally, a Straube tail phenomenon; convulsions did not occur. In the guinea pig, a clonic seizure pattern similar to that in the mouse was observed following intracerebral injection of histamine in doses of 5-10 mg/kg. A dose of 10 mg/kg produced seizures consistently and was used in subsequent tests of anticonvulsant activity of drugs. Compared to the mouse, occurrence of convulsions and death was more rapid; generalized clonus began within 10 to 60 sec. and the animals died in 1 to 4 min. A typical seizure in the guinea pig consisted of cycling movements of fore-limbs followed by generalized clonus; tonic extension of hind-limbs was noted rarely. While the convulsive and lethal intracerebral dose of histamine in the guinea pig was smaller than in the mouse, the difference in sensitivity was considerably greater when histamine was injected intraperitoneally. By this route of administration, the $LD_{97}$ in the guinea pig and mouse was 10 and 2500 mg/kg, respectively.

*Actions of drugs on histamine-induced seizures. Anticonvulsants.* (Table I). Trimethadione and phenobarbital abolished seizures completely and the animals survived; protective or therapeutic indices in the mouse compared favorably with those reported in

TABLE I. Effect of Anticonvulsants against Seizures Induced by Intracerebral Histamine.

| Drug | Time of maximal effect, hr | Effective $dose_{50}$, mg/kg | Protective index* |
|---|---|---|---|
| Trimethadione | 1½ | 295<br>(204–428) | 2.3 |
| Phenobarbital | 3 | 30<br>( 20– 44) | 1.9 |
| Acetazolamide | 3 | 630†<br>(460–860) | 3.2 |
| Diphenylhydantoin | 3 | — ‡ | 0 |

* Ratio $TD_{50}/ED_{50}$. † Incomplete control.
‡ Seizures exacerbated.

tests of anti-metrazol activity(13). In the sedative doses employed, both trimethadione and phenobarbital were effective also against histamine seizures in the guinea pig. Respective values for the $ED_{50}$ were not determined in this species. The depressant effect of trimethadione was remarkable in the guinea pig and much greater than in the mouse. In mice, acetazolamide in non-toxic doses prevented severe clonic seizures and death in tonus; hyperkinesia, circling movements, and mild clonic jerks were not abolished, even with doses of 1800 mg/kg. In guinea pigs, the partial anticonvulsant action of acetazolamide observed in mice was not demonstrated at a dose level of 1800 mg/kg. Diphenylhydantoin, 25-300 mg/kg in mice and 50 mg/kg in guinea pigs, failed to prevent and indeed, exacerbated histamine-induced clonic seizures; the small tonic component of the seizure was abolished but death ensued.

*Tranquilizers.* Chlorpromazine, in oral doses of 5 and 50 mg/kg, had no anticonvulsant or antilethal action in mice tested with histamine intracerebrally after an interval of 1 hr. In guinea pigs given chlorpromazine intraperitoneally and tested at ½ hr, death from intracerebral histamine was prevented by a dose of 5 mg/kg and the seizure was obtunded in addition in animals treated with a dose of 25 mg/kg. Injection of histamine was repeated after 2 hours in 5 animals given the larger dose of chlorpromazine; 2 animals exhibited generalized clonic seizures of delayed onset but all survived. Both mice and guinea pigs were sedated by the larger doses of chlorpromazine employed. In addition to a protective effect against histamine administered intracerebrally, chlorpromazine, in a dose of 25 mg/kg, prevented convulsions and death in guinea pigs given 3 lethal doses of histamine (30 mg/kg) by the intraperitoneal route. Mice treated with the same dose of chlorpromazine were not protected from 2 lethal doses of histamine (6000 mg/kg intraperitoneally) and, compared with controls, the time of death was accelerated. Serpasil, in sedative doses of 20 and 40 mg/kg, caused exacerbation of seizures in both mice and guinea pigs; animals were challenged with histamine at 1½ hr after administration of drug.

*Antihistaminics.* In mice tested at 1, 2 and 3 hr after oral administration of drug, tripelennamine 50-150 mg/kg, diphenhydramine 25-50 mg/kg, and thephorin 100-200 mg/kg failed to protect against histamine-induced seizures and death. Tripelennamine, in toxic doses of 200 and 250 mg/kg, prevented convulsions and death in 2 of 4 mice tested with intracerebral histamine; larger doses of 400-500 mg/kg had convulsant and lethal effects. Diphenhydramine, in 100, 200 and 300 mg/kg doses and thephorin, 400 and 500 mg/kg, caused hyperkinesia, convulsions and death in tonus. In guinea pigs tested at 10-15 min. after intraperitoneal administration of drug, antihistaminics showed protective effects. Tripelennamine, in a non-toxic dose of 5 mg/kg, prevented death and reduced severity of histamine-induced seizures. At a dose of 10 mg/kg, both tripelennamine and diphenhydramine abolished the generalized clonic component of the seizure in 4 of 5 animals tested. Of the 5 guinea pigs treated with diphenhydramine and given a second injection of histamine after an interval of 1 hr, generalized clonic convulsions occurred in 4 and tonic extensor spasms of both fore- and hind-limbs were associated in 2. In the experiment designed to compare the relative potency of effect of antihistaminics in guinea pigs and mice, equal and non-excitant doses of tripelennamine, diphenhydramine and the weak antihistaminic, chlorpromazine showed greater and and more specific anticonvulsant and anti-lethal activity in the guinea pig (Table II). Apart from tripelennamine, which pro-

TABLE II. Comparison of Effects of Antihistaminics in Guinea Pigs and Mice Treated with an Intracerebral $CD_{97}$ of histamine.

| Antihistaminic (25 mg/kg) | Anticonvulsant potency* |  | Anti-lethal potency* |  |
|---|---|---|---|---|
|  | Guinea pig | Mouse | Guinea pig | Mouse |
| Tripelennamine | 2 | 1 | 4 | 1 |
| Diphenhydramine | 3 | 0 | 5 | 2 |
| Chlorpromazine | 3 | 0 | 3 | 2 |
| Total | 8/15<br>(53%) | 1/15<br>(7%) | 12/15<br>(80%) | 5/15<br>(33%) |

* Expressed as No. of animals out of 5 protected.

tected only 1 of 5 mice from histamine-induced seizures, antihistaminics were ineffective as anticonvulsants in this animal species. Indeed, in mice treated with diphenhydramine, the onset of convulsions was accelerated and clonus was exacerbated.

*Adrenocortical steroids.* Cortisone acetate, in a dose of 140 mg/kg daily, failed to prevent and accelerated convulsions and death in 10 mice tested after treatment for 4 consecutive days. Desoxycorticosterone acetate, 27 mg/kg administered daily for 4 days, also failed to prevent, but retarded the onset of seizures and death. The threshold to minimal electroshock seizures is affected similarly by adrenocortical steroids(14) and, like histamine-induced seizures, the minimal electroshock seizure though less severe is principally clonic in type. Histamine given intraperitoneally in doses of 5, 50, 500 and 2000 mg/kg delayed the onset of metrazol-induced seizures by 3.6, 2.2, 6.1 and 24 min., respectively. In 10 control mice, mean time of onset of clonus was 2.9 min. (with a range of 1.5-4.5); in 4 groups of 5 histamine-treated mice, clonus occurred at 6.5, 5.1, 9 and 27 min., respectively. Generalized clonic seizures were prevented in 1 of 5 animals given histamine in a dose of 500 mg/kg and in a similar number treated with a toxic dose of 2000 mg/kg. Histamine, by intraperitoneal injection in the same dose range, had no significant effect on the maximal electroshock seizure threshold in mice.

*Discussion.* When administered intraperitoneally, histamine will induce seizures fairly consistently in the guinea pig but less commonly in the mouse. In response to intracerebral histamine, however, seizures are the most remarkable manifestation of toxicity and their properties may be compared in both animal species. Histamine-induced seizures are principally clonic in type and the pattern resembles that produced by metrazol and by fever(7). They are prevented by trimethadione, an antiepileptic drug which controls experimental metrazol and febrile seizures, and are exacerbated by diphenylhydantoin, which is ineffective against metrazol and fever-induced seizures(7). Whereas trimethadione is of value clinically in treatment of petit mal and minor clonic seizures, the incidence of such seizures may be increased by diphenylhydantoin(4). Evidence of a diencephalic origin for petit mal(15) and possibly for febrile seizures(16) has been reported. Further, it is of interest that relatively high concentrations of histamine have been found in the region of the hypothalamus(1). These observations, together with the known efficacy of antihistaminics in treatment of petit mal, suggest that paroxysmal and excessive release of histamine in the diencephalon may possibly be involved in the origin of certain seizure discharges.

That histamine may be involved in the seizure mechanism is supported further by the anticonvulsant action of antihistaminics observed in laboratory animals. In the guinea pig, an animal highly sensitive to histamine, tripelennamine and diphenhydramine are antagonistic to histamine-induced seizures whereas in the mouse, a histamine-resistant species, antihistaminics have little or no protective effect. The high natural resistance observed when histamine is administered parenterally in the mouse is not understood; it may be explained by a relatively rapid rate of inactivation by acetylation, cellular binding and the enzyme histaminase. The minimal signs of toxicity to the central nervous system may be due to low concentrations of drug in the brain. The difference in sensitivity between mouse and guinea pig is much less remarkable when histamine is given by direct intracerebral injection. While pertussis vaccine is known to enhance sensitivity of the mouse to histamine given intraperitoneally (17,18) an exacerbation of convulsions induced by histamine given intracerebrally could not be demonstrated.§ However, despite the greater uniformity in sensitivity to intracerebral histamine, the guinea pig and mouse differ markedly in response to antihistaminics. In addition to their ineffectiveness against histamine-induced seizures in the mouse, antihistaminics fail to protect this species from a lethal parenteral dose of histamine. In the mouse, toxic effects of antihistaminics are added to those of histamine (19) whereas in the guinea pig, protective effects are evident. The ability of chlorpromazine to obtund histamine seizures in the guinea pig but not in the mouse may be related to its weak antihistaminic activity. Serpasil, a tranquilizing agent without antihistaminic properties, exacerbated histamine seizures in both mice and guinea pigs.

The guinea pig differs from the mouse and rat, not only in its sensitivity to histamine but in the seizure response to electroshock stimuli. A maximal seizure with tonic extension of hind-limbs is observed rarely and generalized clonus is the principal component (20). Since histamine-induced seizures are mainly clonic in type and certain clinical convulsive disorders may possibly be related to abnormal histamine release, the guinea pig should prove of value in laboratory evaluation of potential anticonvulsant compounds.

Libet *et al.*(21) have investigated a possible *anticonvulsant* action of histamine given parenterally in dogs. Seizure discharges induced by leptazol were prevented or their onset was delayed. The apparent anticonvulsant effect was explained by histamine-induced vasodilation and consequent dilution of leptazol. The present experiments in mice confirm these observations. While metrazol-induced seizures are retarded or abolished by histamine injected intraperitoneally, the threshold to electroshock seizures is not modified significantly. Thus, a non-specific antagonism to metrazol and seizures is suggested. Furthermore, in clinical studies, histamine failed to control petit mal seizures (22) and had no demonstrable effect on the electroencephalogram(23).

*Summary.* Clonic seizures were induced in the mouse and guinea pig by intracerebral injection of histamine. They were prevented by trimethadione, exacerbated by diphenylhydantoin, and incompletely controlled by acetazolamide. Apart from chlorpromazine, tranquilizing agents were ineffective. Cortisone and desoxycorticosterone caused mild exacerbation and some reduction in severity of seizures, respectively. Antihistaminics prevented histamine seizures in the guinea pig but not in the mouse. The difference in sensitivity of these species to histamine by intraperitoneal injection was less evident by the intracerebral route. Control of metrazol-induced seizures by histamine was a non-specific antagonism. The possible relation of histamine to seizure mechanisms is discussed.

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## Comparative *in vitro* Corticoidogenic Effects of Rat Adenohypophysial Extract and U.S.P. Reference Standard Corticotropin.* (24441)

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Consistently higher values for pituitary ACTH concentration, in the intact rat, have been obtained with Saffran and Schally's *in vitro* corticoidogenic assay(1) than with Sayers' *in vivo* adrenal ascorbic acid depletion method(2). Due allowances being made for the different strains of rats and extraction procedures utilized by various groups, the values recorded, in terms of milliunits (mu) of ACTH/mg of fresh pituitary tissue, range from 10 to 18(3-6) with Sayers', and from 52 to 241(7,8) with Saffran and Schally's assay. A mean concentration of 64.6 ± 1.8 mu/mg was recorded, in this laboratory, from 100 *in vitro* assays (average $\lambda$ : 0.106 ± 0.004) of 0.1N HCl-extracted pituitaries from intact male Sprague-Dawley rats (Fortier, unpublished). To account for the discrepancy in results yielded by the 2 assaying procedures, the possibility was raised by Birmingham *et al.*(7) that *steroidogenic/ascorbic acid depleting* (S/AA) activity ratio might be higher in native (pituitary extract) than in more purified (Reference Standard) corticotropin. This view was not supported by results of concurrent 4-point-2-dose assays of the same pituitary extract against the same standard, respectively based on *in vitro* corticoidogenesis, and on adrenal ascorbic acid depletion and rise of adrenal and plasma corticosteroids in hypophysectomized rats (Guillemin, Fortier and Lipscomb, unpublished). Nearly identical values (20-24 mu/mg) were obtained on the basis of *in vivo* corticoidogenesis and ascorbic acid depletion, whereas a markedly higher concentration (62 mu/mg) was again recorded on the basis of *in vitro* corticoidogenesis. The discrepancy would thus appear to be related to differences in the nature (*in vivo* or *in vitro*) rather than in the end-points (adrenal ascorbic acid depletion or corticoidogenesis) of the assays. The following possibilities were accordingly considered in the present study: a) Conditions prevailing in the *in vitro* assay might favor activation of a structurally related, hypothetical precursor of ACTH (*protocorticotropin*), contained in rat pituitary extract; b) Differences in the fates of, or responses to, the presumably distinct molecules corresponding to rat and hog (U.S.P. Reference Standard) corticotropins might be selectively enhanced by conditions prevailing in one of the 2 assaying systems under consideration. These possibilities were assessed by comparing rat pituitary extract and U.S.P. Reference Standard Corticotropin, in Saffran and Schally's *in vitro* system, with respect to inactivation, corticoidogenic effects, and extent of corticotrophic activation conferred to the incubated glands, as a result of a limited period of contact.

*Materials and methods. Pituitary extracts.* Adenohypophyses from adult male Sprague-

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Dawley rats, killed by decapitation, were quickly removed from the skull, separated from neural lobe, wiped clean of blood, and weighed to nearest 0.05 mg. Pooled glands from 3 rats were ground with a few grains of sand in 15 ml conical centrifuge tube containing 0.6 ml of 0.1N HCl. The rod was rinsed into tube with 0.3 ml of 0.1N HCl. After 30 minutes at room temperature, the stoppered extract was stored in deep-freeze. A weighed sample of U.S.P. Reference Standard Corticotropin was dissolved in equinormal HCl, and stored for same period as the extract. On day of assay, both were thawed, and adjusted to 0.01 normality. *Comparative effects of incubation on in vitro corticoidogenic activity of U.S.P. Reference Standard Corticotropin and rat pituitary extract.* Aliquots of U.S.P. Reference Standard Corticotropin, corresponding to 10 ($U^1$) and to 30 ($U^2$) mu of ACTH, in 0.3 ml of solvent, /100 mg adrenal, were equally distributed among 4 of a set (A) of 8 beakers containing 1.4 ml of Krebs-Ringer-Bicarbonate-Glucose medium. These beakers were incubated for 2 hours in Dubnoff Metabolic Shaking Incubator, at 38°C and in water-saturated atmosphere of 95% $O_2$, 5% $CO_2$. At end of this incubation period, corresponding to end of concurrent one-hour preincubation of another set (B) of 8 beakers containing quartered adrenal glands in the same volume of medium, similar aliquots ($S^1$ and $S^2$) of fresh ACTH were added to the 4 remaining beakers of set A, and the glands rapidly transferred from beakers of set B to those of set A. Incubation was resumed for additional 2 hours, and potency ratio of incubated to fresh material assessed by means of Bliss' methods for factorial analysis and analysis of variance(9), on the basis of their respective effects on *in vitro* production of UV-absorbing steroids(1). In analogous experiments, aliquots of fresh and incubated rat pituitary extract, corresponding to one ($S^1$ and $U^1$) and to 3 ($S^2$ and $U^2$) fiftieths of a gland, in a volume of 0.3 ml, /100 mg adrenal, were substituted to those of Reference Standard Corticotropin. In both instances, actual weights of glands with which they would eventually be incubated being unknown by the time the U aliquots were added, the doses/100 mg adrenal were determined on basis of average adrenal weight of 2 rats of the same B.W. as those to be killed for assay one hour later. The S doses of fresh material were adjusted for each beaker, by multiplying true weight of glands therein contained by the ratio: *assumed adrenal weight/true adrenal weight,* in corresponding U beaker. *Comparative in vitro corticoidogenic effects of U.S.P. Reference Standard Corticotropin and Rat Pituitary Extract.* Quartered adrenal glands from 48 female Sprague-Dawley rats (120-130 g, B.W.) were distributed, in separate experiments, among 6 homogeneous sets of 8 units, each made up of 8 quarters from same donor population. These were weighed to nearest 0.05 mg, and allotted to beakers containing 1.5 ml of freshly gassed medium. Following preincubation for one hour, the fluid was aspirated off and replaced with 1.4 ml of fresh medium. In 4 of the 6 sets, 20 mu of Reference Standard Corticotropin, in 0.3 ml of solvent, /100 mg adrenal was added to one-half of the beakers; a similar volume of extract, corresponding to 0.4 mg of fresh pituitary tissue,† /100 mg adrenal, to the other half. In the remaining 2 sets, only the solvent (0.01N HCl) was added. Incubation was resumed for periods of 15, 30, 60 and 120 minutes (for 2 units of each set), at the end of which 1 and 0.1 ml of the media were respectively used for determination of the UV-absorbing compounds by spectrophotometric method of Saffran and Schally(1), and of the corticosterone-like steroids by the method of Silber, recently described by Guillemin *et al.* (10, 11), and based on the sulfuric acid-induced fluorescence of these steroids(12). *Effect of time of contact with incubated glands on relative potency of rat pituitary extract to U.S.P. Reference Standard Corticotropin.* Following concurrent preincubation, under usual conditions, of 2 sets (A and B) of 8 beakers containing 8 adrenal quarters from the same donor population, the fluid was aspirated off and replaced with 1.4 ml of fresh medium. Aliquots of 10 ($S^1$) and 30 ($S^2$) mu/100 mg adrenal of the reference standard,

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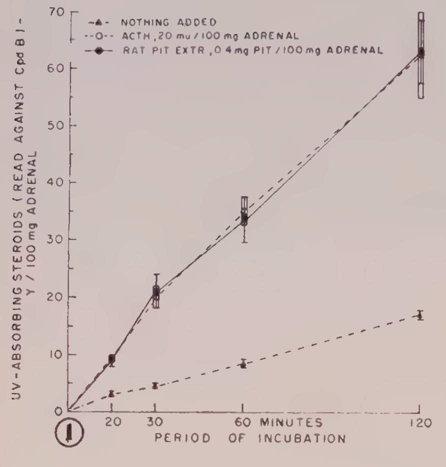
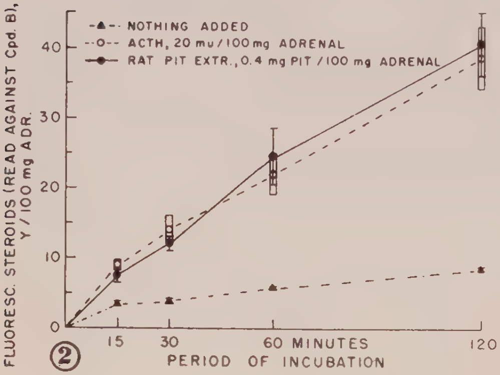
† In preliminary assay of extract, this amount of pituitary tissue was observed to correspond to 20 mu of ACTH.





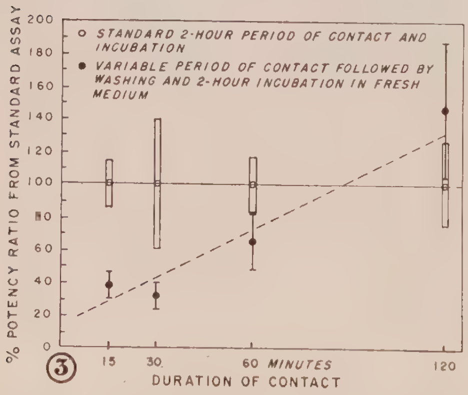


FIG. 1. Comparative *in vitro* corticoidogenic effects, in terms of UV-absorbing steroid production, of U. S. P. Reference Standard Corticotropin and rat pituitary extract. In this, as in following figures, stand. errors are indicated by T-shaped bars and columns.

FIG. 2. Comparative *in vitro* corticoidogenic effects, in terms of fluorescent steroid production, of U. S. P. Reference Standard Corticotropin and rat pituitary extract.

TABLE I. Effect of Incubation for 2 Hours in a Cell-Free Medium on *In Vitro* Corticoidogenic Activity of U.S.P. Reference Standard Corticotropin and Rat Pituitary Extract.

| Sample | Potency ratio* (incubated /fresh) | 95% confidence limits† | Index of precision (λ) |
|---|---|---|---|
| ACTH, U.S.P. ref. std. | .959 | .780-1.175 | .044 |
| Rat pituitary extr. | .974 | .294-3.039 | .174 |
| | .813 | .660- .986 | .043 |

* Antilog M. † Antilog $C^2M \pm CtSm(9)$.

in 0.3 ml of solvent, were added to 4 of the beakers of each set; doses of pituitary extract, corresponding to one ($U^1$) and to 3 ($U^2$) fiftieths of a gland, to the other 4. In 4 successive experiments of this type, incubation was resumed for a period of 2 hours for the A sets, of 15, 30, 60 and 120 minutes respectively, for the B sets. The media from the latter were aspirated off and discarded. The glands were twice washed with fresh medium, 1.5 ml of which was added, prior to a final 2 hour-incubation. Potency ratios from standard (A) and modified (B) assays were determined as usual, and the effect of variable periods of contact, ascertained from their differences.

*Results.* Little alteration of the *in vitro* corticoidogenic activity of U.S.P. Reference Standard Corticotropin or rat pituitary extract resulted from their respective incubations for 2 hours in a cell-free medium‡ (Table I). The closeness of the potency ra-

FIG. 3. Effect of duration of contact with the incubated glands on potency ratio of rat pituitary extract to U. S. P. Reference Standard Corticotropin, determined on basis of residual corticotrophic activation conferred to the glands. Of the 2 concurrent assays run for each time ordinate, one was carried out according to standard procedure; the corticotrophic principles remaining in contact with the glands throughout the 2-hr period of incubation, the second involved a limited period of contact, followed by washing of the glands, prior to resumption of incubation for 2 hr in fresh medium. Results are expressed as percentages of potency ratios from the corresponding standard assays. Stand. errors were derived from Sm's of the assays.

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‡ Partial (44%) loss of activity of the reference standard, previously reported(13) as a result of incubation, is imputed to neutralization of the sample, prior to incubation.

tios and overlap of their confidence limits would seem to preclude, furthermore, a selective effect of incubation on 1 of the 2 corticotropins. Both corticotropins markedly enhanced the basal production of UV-absorbing (Fig. 1) and fluorescent (Fig. 2) steroids by the incubated glands. From the parallelism of the slopes, contiguity of the means, and overlap of the standard errors, the *in vitro* corticoidogenic effects of reference standard and pituitary extract appeared to be qualitatively and quantitatively identical, at their respective dosage levels. When, however, relative potency of extract to standard was ascertained in terms of corticotrophic activation conferred to the glands, as a result of a limited period of contact (Fig. 3), it was shown to increase linearly with duration of contact.

*Discussion.* *In vitro* activation of a hypothetical *protocorticotropin* in the pituitary extract would have been expected to result in increasing divergence, with time, of the corticoidogenic responses to the extract and to the reference standard. This possibility is excluded by the observed parallelism of the responses. Differences in rates of *in vitro* inactivation of the 2 principles, as a result of incubation in a cell-free medium or in the presence of the glands, are likewise precluded by the similarity of their *incubated/fresh* potency ratios, and the lack of divergence of their time-response slopes throughout incubation.

The UV-absorbing and fluorescent properties of the steroids, used in this study as indices of *in vitro* corticoidogenic activity, have been respectively ascribed to the Δ 4-3-ketone group(14,15) and, on the basis of indirect evidence(16), α-ketolic side chains.§ The strict comparability of the corticoidogenic effects of extract and standard, ascertained in terms of either type of steroids, provides further evidence of their similarity with respect to fate and mode of action in the *in vitro* system. However, the increase, with time of contact, of the corticotrophic activation conferred to the glands by the extract, relatively to the standard, points to a difference. If the residual activation of the corticosteroidogenic process, which persists after withdrawal of ACTH from the medium, is related, as appears likely, to the amount of hormone already fixed or adsorbed by the incubated adrenals(17), this finding could be explained in terms of different rates of adsorption, by the glands, of 2 distinct molecules of corticotropin. Such a difference would be masked by the negligible rate of ACTH inactivation observed in the *in vitro* system, but would have a definite bearing on relative potency of the 2 corticotropins under conditions of rapid inactivation or disappearance from the medium, such as those prevailing *in vivo*(18). Assuming identical rates of inactivation or disappearance of the 2 molecules, under these conditions, their potency ratio would be conditioned, at least partly, by their respective rates of adsorption. There may lie the explanation for the divergent estimates of rat pituitary ACTH concentration yielded by *in vivo* and *in vitro* assays.

*Summary.* Rat pituitary extract and U.S.P. Reference Standard Corticotropin were compared, in Saffran and Schally's *in vitro* system, with respect to inactivation, corticoidogenic effects, and corticotrophic activation conferred to the incubated glands, as a result of a limited period of contact. The only difference noted pertained to the extent of this residual activation. The relative potency of pituitary extract to reference standard, determined on that basis, was observed to increase linearly with time of contact. This is ascribed to different rates of adsorption, by the glands, of the 2 corticotropins. Taken in conjunction with the more rapid inactivation and disappearance of ACTH *in vivo* than *in vitro*, differential adsorption is suggested as the basis of the discrepancy in the estimates of rat pituitary ACTH concentration, yielded by *in vivo* and *in vitro* assays.

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§ Other characteristics are, no doubt, involved in the fluorescence reaction(12). However, α-ketolic side chains admittedly accounting for blue tetrazolium reduction, production ratios of fluorescent to UV-absorbing steroids by adrenal glands, incubated in presence or absence of ACTH, were observed to correspond to the previously recorded(14) *blue tetrazolium reducing/UV-absorbing* ratios under comparable conditions.

The author is indebted to Miss R. C. Wood for able technical assistance.

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## Effect of Pantothenic Acid on the Longevity of Mice. (24442)

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The disparity between the life span of worker and queen bees suggested the possibility that pantothenic acid which is the most conspicuous nutritional constituent of royal jelly(1,2) might have the effect of prolonging life in other species. An additional suggestion came from the finding of Gardner(3) that this vitamin acts as an anti-ageing factor in drosophila.

*Method.* Thirty-four male and 40 female C-57 black mice, age 4-5 weeks, were procured from the Jackson Memorial Laboratory. The mice were grouped with either 6 or 7 animals of the same sex in each cage. Thirteen male and 20 female mice were given supplementary pantothenic acid and 21 male and 20 female mice were used for the control group. All mice were fed Purina laboratory pellets throughout the experiment. It was desired to give each of the 33 mice in the experimental group a supplement of approximately 300 μg of calcium pantothenate per day throughout the experiment. The vitamin was added to the drinking water and the amount added was based on the previous week's water consumption, *i.e.,* if average daily water consumption for the group in one cage was 4 ml per mouse, calcium pantothenate was added in the amount of 75 μg/ml. If the consumption was 5 ml then 60 μg of the vitamin were added per ml of water. The age of each mouse was

TABLE I. Summary and Statistical Data for Mice Which Received Pantothenic Acid Supplement and Controls.

| | Mean life span in days | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mice with pantothenic acid supplement | | | Controls | | | t test(4) | U test(5) |
| | No. | Days | S.D. | No. | Days | S.D. | P = | P = |
| Both sexes | 33 | 653.1 | 201 | 41 | 549.8 | 233 | .05 | .01 |
| Male mice | 13 | 660.4 | 220 | 21 | 559.0 | 167 | .15 | .04 |
| Female mice | 20 | 648.4 | 187 | 20 | 540.2 | 285 | .18 | .05 |

TABLE II. Analysis by Sex of Mice Given Pantothenic Acid Supplement and Control Mice.

| | Mean life span in days | | | | Days difference ♂ & ♀ | % difference ♂ & ♀ |
|---|---|---|---|---|---|---|
| | No. | ♂ | No. | ♀ | | |
| Mice with pantothenic acid supplement | 13 | 660.4 | 20 | 648.4 | 12.0 | 2 |
| Controls | 21 | 559.0 | 20 | 540.2 | 18.8 | 3 |
| Days difference treated & controls | | 101.4 | | 108.2 | | |
| % difference treated & controls | | 18% | | 20% | | |

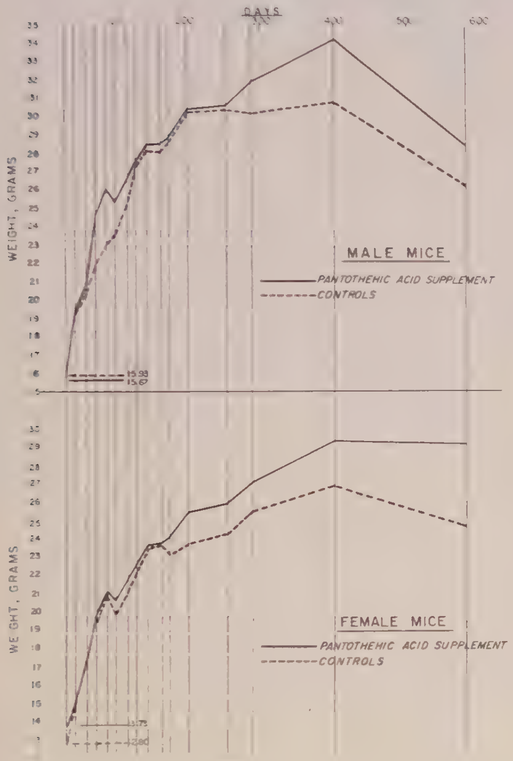

FIG. 1.

taken arbitrarily as 35 days at start of experiment.

*Results.* The data obtained are given in Tables I and II.

The above analysis (Table II) shows that differences in life span, 2% and 3% between treated males and females and control males and females, respectively, are negligible. There was however, a difference of 18% in life span betwen treated and untreated males and a difference of 20% between treated and untreated females.

The mice were weighed at 2-week intervals for the first 6 months and at greater intervals for the following year (Fig. 1, vertical lines). Both male and female mice which received pantothenic acid supplement gained and maintained slightly greater weight after about 250 days than did control mice. It is speculative as to whether this greater weight should be interpreted as indicative of better general health or as a sign of prolonged pre-senile period. The results of this experiment seem sufficiently significant to suggest further work with other species.

*Summary.* 1) Thirty-three young male and female C-57 black mice were given approximately 300 μg of calcium pantothenate daily in drinking water. Forty-one control mice did not receive the vitamin supplement. 2) The mean life span for the mice given supplementary calcium pantothenate was 653.1 days and that for the control mice was 549.8 days. The statistical difference between the 2 groups is P = 0.05 (T test) 0.01 (U test). 3) The mice which received the vitamin supplement maintained slightly greater body weight after they were approximately 250 days old.

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# Lack of Normal Blood Pressure Rise in Growing Chicken Deprived of Choline in Early Life.* (24443)

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Experimental production of persisting arterial hypertension has not been accomplished in the chicken. In the present experiment the method of choline deprivation was adopted for this purpose. In the rat this method results in arterial hypertension(1).

*Methods.* One-day-old Hyline cockerels were fed a synthetic diet devoid of choline (Table I). Controls kept in the same brooder were fed a commercial chick starter mash (Kahn Bros.). After 5 weeks the choline-deprived chicks were transferred to starter mash. Later a group of these birds was subjected to another period of choline deprivation of 8 weeks, then put back on starter mash. Following the initial transfer of choline-deprived chicks to starter mash, they were weighed at approximately monthly intervals and arterial blood pressures determined by needle puncture of sciatic artery in unanesthetized birds. Pressures were recorded with Sanborn transducer and recorder. Several choline-deprived and control chicks were killed at end of each day of blood pressure determination. Hearts, kidneys and testes were saved. Comb indices were computed by multiplying greatest height times greatest length of comb in centimeters.

*Results.* After 5 weeks on synthetic diet, the choline-deprived chicks were weak and underweight in comparison to mash-fed controls (145-300 g compared to 355-460 g for controls). They had badly swollen hock joints, suggesting chick perosis. When these chicks were transferred to starter mash, they recovered normal weight and appearance within 3 months. A second period of choline deprivation in some birds resulted in same signs within 8 weeks and was similarly followed by recovery when chicks were fed mash. Table II summarizes blood pressure findings. Choline-deprived chicks showed no increases in blood pressure through the seventh month after initial transfer to starter mash.

TABLE I. Composition of Choline-Deficient Diet.

| | |
|---|---|
| Drackett C-1 assay protein (Soy) | 1412 g |
| Glycine | 12 |
| D-L methionine | 30 |
| Cerelose (glucose) | 2232 |
| USP XIV salt mixture | 214 |
| Mixed water soluble vitamins* | 40 |
| " oil " " † | 60 |

* Two kg of this mixture contained, in g, the following amounts of vitamins: thiamine 100, nicotinic acid 100, riboflavin 16, calcium pantothenate 20, vit $B_{12}$ 0.02, pyridoxine HCl 6, biotin 0.6, folic acid 4, inositol 100, para-amino benzoic acid 2, ascorbic acid 250.

† Two kg of this mixture, in cottonseed oil, contained, in g: vit K 5, vit E (Ephynal acetate) 20, and in units, vit A 10,000,000 and vit D 600,000.

Control chicks, on the other hand, showed rises in blood pressure after the third month. Testes of choline-deprived chicks, autopsied 6 months after transfer to mash weighed only one-fourth as much as those of control chicks (Table III). Testicular function, as measured by comb size, was also depressed. By comparison, kidney weights were the same or higher than in control birds. Microscopic examination of kidneys from several choline-deprived birds at various times following transfer to mash diet, revealed presence of partly hyalinized glomeruli and of tubules and collecting ducts which had undergone cloudy swelling, vacuolization, and desquamation of epithelial linings. Changes found in kidneys did not appear to be useful in explaining the blood pressure findings.

*Discussion.* The present data demonstrate that cockerels deprived of choline early in life do not become hypertensive when subsequently fed a diet adequate in choline, but instead remain normotensive and do not show the usual rise in blood pressure with age(2). Depressed growth of testes and combs indicates that either lack of androgens or pituitary gonadotrophins is responsible for this

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TABLE II. Mean Blood Pressure in Choline-Deprived Cockerels (mm Hg).

| | Months after end of initial choline-deficient period | | | | | | |
|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 6 | 7 |
| Controls | 138 ± 5.7*<br>(7)† | 141 ± 3.8<br>(5) | | 139 ± 5.1<br>(5) | 150 ± 7.1<br>(5) | 168 ± 5.7<br>(11) | 173 ± 6.4<br>(6) |
| Choline-def. | 157 ± 4.6<br>(7) | 149 ± 4.3<br>(5) | 152 ± 3.7<br>(5) | 150 ± 6.5<br>(5) | 144 ± 4.8<br>(5) | 147 ± 10.5<br>(8) | 157 ± 8.8<br>(7) |
| Repeat choline-def. | | | | 146 ± 3.8<br>(5) | 133 ± 5.8<br>(5) | 130 ± 5.9<br>(4) | |

* Mean ± S.E.
† No. in parentheses refer to No. of determinations, one determination/bird each month.

phenomenon. Male chicks and capons, but not females, are said to show increases in blood pressure with age after puberty, and this increased pressure in adult males and capons(3) is reduced by suppression of pituitary gonadotrophin secretion by estrogens or drugs(3). These findings indicate that pituitary gonadotrophins rather than androgens are required for normal rise in blood pressure with age.

TABLE III. Chick Kidney and Testis Weights at 6 Months after End of Initial Choline-Deficient Period.

| | Wt of testes, g/100 g body wt | Comb index, CM² | Wt of kidneys, g/100 g body wt |
|---|---|---|---|
| Controls | .38 ± .10*<br>(5)† | 128 ± 8.3<br>(5) | .85 ± .07<br>(5) |
| Repeat choline-def. | .10 ± .01<br>(4) | 84 ± 8.9<br>(4) | 1.06 ± .08<br>(4) |

* Mean ± S.E. † No. of birds.

*Summary.* It is concluded that in the present experiments lack of rise in blood pressure with age in choline-deprived chicks was due directly to suppression of pituitary gonadotrophins secretion caused by lack of choline in early weeks of life, and not to consequent lack of androgens.

The author wishes to express his thanks to Mr. Anthony Ellis for technical assistance.

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# Parasitic *Philornis* Flies as Possible Sources of Arbor Virus Infections (Diptera, Anthomyidae).* (24444)

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(Introduced by Jordi Casals)
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In the course of a nestling bird bleeding program conducted in Trinidad as part of a general study of arthropod-borne (ARBOR) viruses, it has been observed that many nestlings are infected with immature fly parasites. Some of the early reared material was sent to Dr. Curtis Sabrosky at U. S. National Museum in Washington, D. C., who identified the flies as belonging to genus *Philornis* Macquart in the family Anthomyidae. They are thus biologically similar to North American bird parasites of the genus *Apaulina* Hall and *Protocalliphora* Hough of the Old World, family Calliphoridae(1). Continuing observations soon made it evident that we were dealing with several species of *Philornis* and that various degrees of parasitism were involved (including subcutaneous and external parasitism). All early material was reared out for identification and is being studied by Dr. H. R. Dodge, who is revising the genus. Now we are also becoming interested in the potentialities of *Philornis* as possible virus carriers, and are experimenting with much of the material presently being obtained. The purpose of this note is to record certain preliminary observations made along these lines.

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* Studies and observations upon which this paper is based were conducted in the Trinidad Regional Virus Lab. with the support and under auspices of Government of Trinidad and Tobago, Colonial Development and Welfare Scheme, and Rockefeller Fn.

On 16 July, 1957, a communal nest of anis (*Crotophaga ani*) was seen at Vega de Oropouche in which 4 of the 6 nestlings were infested with several *Philornis* larvae (designated by us "species F"). These were external parasites feeding on host by rasping the skin with oral hooks and ingesting the oozing serous fluid and blood. The same day in the laboratory, the birds were inoculated intramuscularly with 0.1 ml of a $10^{-4}$ dilution (868 $MLD_{50}$) of St. Louis virus (TRVL 9404 strain, pool #3). One *Philornis* larva which was placed on bird #882 20 July (fourth postinoculation day) and allowed to feed overnight, dropped the following morning and was ground up and inoculated into a group of 6 adult mice 0.03 ml i.c. A 2/6 mortality ratio resulted, with deaths on days 6 and 7. The agent was isolated in further passage and confirmed as St. Louis virus in a specificity test using a known St. Louis immune serum. Circulating virus was demonstrated in the host bird on 21 July (fifth postinoculation day), when there was a titer of $10^{-3.5}$. A specificity test confirmed its identity with St. Louis virus.

On 18 March, 1958, 22 larvae of *Philornis* "species G" were expressed from under the skin of 2 week-old nestling doves (*Leptoptila* sp.) taken at Fishing Pond. In contrast to the previous fly, species "G" is a subcutaneous parasite. The same day 12 of these larvae were inoculated by glass capillary pipette, each with an estimated 0.002 ml of a $10^{-5}$ dilution of Ilhéus virus (TRVL 10507 strain, pool #5). This pool titered $10^{-8.16}$ in a 0.03 ml intracerebral inoculation of adult mice; calculation of virus titers was made by the method of Reed and Muench(2). The calculated inoculum is 96 $MLD_{50}$. All larvae pupated during the next 2 days. The first adult (female) emerged 28 March after 10 days of pupation (it was also the tenth postinoculation day). This adult (specimen #9) and one pupa (#8) were ground individually in 1 ml of bovalbumin diluent fortified with antibiotics, centrifuged at 8,000 rpm for 10 minutes and inoculated into 2 groups of 6 adult mice 0.03 ml i.c. The following day 7 more adults emerged and were treated similarly. The remaining 3 pupae failed to survive.

Of the 8 adult flies and one pupa tested, 2 flies (#9 and #11) were positive for virus; a specificity test confirmed it to be Ilhéus. As indicated above, these flies incubated the virus for 10 and 11 days before being tested. Virus from fly #9 titered $10^{-5.16}$ and that from fly #11 titered $10^{-1.76}$. When these figures are adjusted for amount of diluent to give a figure for total virus content/fly, we find that the amount of virus recovered from fly #9 was about 4,750,000 $MLD_{50's}$ and from fly #11 about 1,900 $MLD_{50's}$. Since the original inoculum going into the larvae contained approximately 96 $MLD_{50's}$, evidence of multiplication of virus was obtained in both flies. The reason for the low percentage of infected flies is not known, but it may be that the capillary needle became plugged.

A third experiment was initiated 24 April, 1958, when a large number of larvae of *Philornis* "species A" were taken from the nest hole of a jacamar, *Galbula ruficauda*, at Vega de Oropouche. This appears to be a coprophilous species of *Philornis* in that the larvae feed on bird excrement. Fifty of these larvae were inoculated the same day by glass capillary pipette with a $10^{-5}$ dilution of St. Louis virus (TRVL 13375 strain, pool #2) which titered $10^{-6.64}$ in baby mice (intracerebral inoculum of 0.02 ml). The larval inoculum of 0.002 ml is estimated to contain approximately 4 $MLD_{50}$ of virus. All larvae pupated within the next 2 days and adults appeared 9-10 days after pupation, at which time they were stored at –60°C.

Eight adults were subsequently tested for virus. Each was ground individually in 1 ml of diluent (called $10^{-1}$), centrifuged for ½ hour and inoculated into baby mice. Six of the flies proved to harbor virus which killed all the mice in their respective groups. Subsequently a specificity test confirmed the virus to be St. Louis. Titrations were made of 5 flies, and titers of $10^{-2}$, $10^{-2.2}$, $10^{-2.5}$, $10^{-3.8}$ and $<10^{-4.3}$ were obtained. This indicates virus quantities ranging from 3,300 to $>650{,}000$ $MLD_{50}$ per fly.

*Discussion.* The role that *Philornis* flies may play in the epidemiology of certain arthropod-borne viruses, such as St. Louis or Ilhéus, is not known. The evidence presented indicates that flies may become infected as larvae and remain infected on reaching the adult state 10 days later. Inasmuch as the adult fly is not a bloodsucker and a single fly generation is associated with only one nest, some other means must exist for insuring virus survival. Possibly susceptible birds become infected through eating infected flies, or the virus may be transmitted through the fly's egg to its progeny. At the moment these possibilities are still unknowns. Thus far, no naturally infected *Philornis* have been encountered.

*Summary.* Fly parasites of the genus *Philornis* (Anthomyidae) attacking nestling birds have been shown capable of becoming infected with St. Louis and Ilhéus viruses. In one instance, a larva was found positive for St. Louis virus after feeding on a nestling ani that was circulating this virus. In a second case, 2 of 9 adult flies became infected with Ilhéus virus after they had been inoculated as larvae 10 and 11 days previously. Similarly, 6 of 8 adult flies proved positive for St. Louis virus after being inoculated 10 days previously as larvae.

We are grateful for the advice of Dr. Richard M. Taylor who suggested the inoculation of *Philornis* larvae.

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# Effect of Autonomic Blocking Agents on Depot Fat Mobilization in Normal and Adrenalectomized Animals.* (24445)

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Hausberger(1) showed that the functional activity of fat cells was regulated by an abundant nerve supply, not only to blood vessels, but to parenchymal cells as well. Trophic influence of the autonomic nervous system on fat tissue has since been demonstrated by several other investigators(2-4). Wool *et al.*(5) using liver fat as index of fat mobilization in normal and adrenal demedullated animals, were able to reduce degree of fat accumulation in liver by pretreatment with ergotamine tartrate. Since the level of liver lipids is a resultant of many processes and does not necessarily mirror depot fat activity, we investigated the rate of fat mobilization from a peripheral depot after treatment with various autonomic blocking agents. The fat depot used was the epididymal fat body of the rat. Our results indicate that certain sympathetic system blocking agents do alter the rat's ability to move fat rapidly from this depot during a fast. To demonstrate this, it was necessary to remove the adrenal glands prior to administration of the blocking drugs.

*Methods.* Male Sprague-Dawley rats weighing 90-110 g were used. In larger animals, the bilateral symmetry of the epididymal fat body disappears. Animals were used

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† In partial fulfillment of requirements for degree of Doctor of Philosophy, Graduate School, Georgetown University. Predoctoral fellow of Nat. Inst. of Neurological Diseases, U. S. P. H. S.

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TABLE I. Effect of Autonomic Blocking Agents on Depot Fat Mobilization (12 Hr Fast).

| Procedure | No. of animals | Depot wt, mg |  | Mobilization |  |
|---|---|---|---|---|---|
|  |  | Initial | Final | mg | mg/100 mg initial depot wt |
| *Intact* |  |  |  |  |  |
| Saline | 17 | 89.8 ± 5.6† | 79.0 ± 5.5 | +10.8 ± 2.2 | +13.0 ± 2.4 |
| Dibenzyline (.2 mg)* | 10 | 98.3 ± 5.3 | 87.7 ± 5.8 | +10.6 ± 1.7 | +11.2 ± 2.0 |
| Hexamethonium chloride (1.25 mg ion)* | 11 | 82.5 ± 5.0 | 74.9 ± 5.1 | + 7.6 ± 1.4 | + 9.5 ± 1.6 |
| Ergotamine tartrate (.5 mg)* | 15 | 97.5 ± 2.1 | 87.0 ± 2.4 | +10.5 ± 2.4 | +11.3 ± 2.3 |
| *Idem* (.25 mg) + Dibenzyline (.1 mg)* | 10 | 94.3 ± 8.1 | 83.2 ± 7.9 | +11.1 ± 2.0 | +14.9 ± 2.8 |
| *Sham adrenalectomy* |  |  |  |  |  |
| Saline | 16 | 71.0 ± 4.5 | 69.9 ± 5.1 | + 1.1 ± 1.6 | + 2.5 ± 2.8 |
| Dibenzyline | 8 | 64.5 ± 2.6 | 66.9 ± 4.3 | — 2.4 ± 2.6 | — 3.1 ± 4.0 |
| Hexamethonium chloride | 9 | 76.9 ± 5.6 | 77.4 ± 5.7 | — .5 ± 2.9 | — .9 ± 3.6 |
| Ergotamine tartrate | 9 | 61.3 ± 6.7 | 60.4 ± 6.5 | + .9 ± 3.1 | + 1.5 ± 4.6 |
| *Idem* + Dibenzyline | 7 | 57.6 ± 4.5 | 53.7 ± 4.5 | + 3.9 ± 1.6 | + 7.0 ± 2.8 |
| *Adrenalectomy* |  |  |  |  |  |
| Saline | 30 | 39.5 ± 2.1 | 29.3 ± 1.9 | +10.2 ± .9 | +26.3 ± 2.2 |
| Dibenzyline | 10 | 61.9 ± 7.0 | 46.2 ± 5.8 | +15.7 ± 2.0 | +25.8 ± 2.0 |
| Hexamethonium chloride | 14 | 45.3 ± 4.7 | 34.3 ± 3.2 | +11.0 ± 3.7 | +22.6 ± 3.7 |
| Ergotamine tartrate | 14 | 51.7 ± 4.4 | 52.6 ± 4.5 | — .9 ± 2.3 | — 1.9 ± 4.2 |
| *Idem* + Dibenzyline | 8 | 53.4 ± 4.8 | 49.2 ± 5.1 | + 4.2 ± .8 | + 8.5 ± 2.0 |

* Per 100 g body wt. † ± S.E. + = Mobilization of fat. — = Deposition of fat.

as their own controls. The first epididymal fat body was removed at onset of fasting and the second at end of this period. The fat pad was removed under ether anesthesia by orchiectomy through a lateral scrotal incision. A fat-free area of the spermatic cord was identified and ligated. The testis with its fat pad was excised. Fat was then dissected from its attachment to the epididymis and testis. Total lipids were extracted and weighed according to Bloor's method(6). The difference between amount of extractable lipid in each depot before and after a 12-hour fast, was used as index of peripheral fat mobilization. Bilateral adrenalectomies were performed under ether anesthesia and these animals were maintained on Purina Rat Chow and 0.9% saline *ad lib.* Adrenalectomized animals were studied on seventh post-operative day. Sham operations were performed in a similar fashion. Ergotamine tartrate was administered in a dose of 0.5 mg/100 g body weight at start of 12-hour fast. Dibenzyline, 0.2 mg/100 g body weight was injected at beginning of the fasting period and again after 6 hours of fast. Hexamethonium chloride was given at a dose of 1.25 mg ion/100 g body weight at start of fasting period. 0.25 mg ergotamine tartrate plus 0.1 mg Dibenzyline/100 g body weight was injected at start of 12-hour fast. All doses were given subcutaneously.

*Results.* In intact animals, the autonomic blocking agents used were ineffective in altering normal rate of fat movement out of the epididymal depot during a 12-hour fast (Table I).

Sham adrenalectomized animals, with no previous blocking drug treatment, showed an almost complete inhibition of fat movement out of the epididymal depot during the short 12-hour fast. This difference from intact controls may have been due to release of adrenal steroids resulting from operative procedure. The autonomic blocking agents had no further effect on this phenomenon (Table I).

Adrenalectomized animals had been shown previously to mobilize relatively more fat from the epididymal depot during a fast than do normal animals(7). Administration of ergotamine tartrate produced a marked inhibition of fat movement out of this depot (Table I). The other blocking agents listed above had no significant effect on this process. When half the dose of ergotamine was used together with half the dose of the inactive Dibenzyline, only partial inhibition of fat mobilization was observed (Table I).

*Discussion.* Regulation of fat mobilization during a fast requires that some signal reaches the fat cell and produces conditions for either a diminution of fat deposition or an augmentation of fat movement out of the cell. This signal could be a blood borne humoral factor such as glucose, or certain fat moieties themselves together with hormones from various sources. There is evidence to support such an hypothesis but this does not seem to be the entire controlling mechanism that matches food intake to depot fat turnover. There are many suggestions in the literature that the nervous system and the epinephrines play a role in this phenomenon(1-4,8). There is further evidence that the adrenal steroids play a "permissive" role for certain functions of the sympathetic nervous system and its neurohumors(9). In experiments described above, a variety of autonomic blocking agents was used in an attempt to interfere with the possible neural patterns governing fat cell behavior.

There are many gaps in our knowledge of the mode and site of action of these drugs. Of those used, ergotamine tartrate is known to be the most potent inhibitor of the glycogenolytic effects of epinephrine. Dibenzyline and hexamethonium chloride apparently have little metabolic activity in this regard. It is of interest, therefore, that only ergotamine tartrate was effective in blocking the movement of fat out of the epididymal depot during a fast. This effect, however, could only be demonstrated in the adrenalectomized animal. A possible explanation for this may lie in the relationship between adrenal steroids and epinephrines. In the absence of steroids, small amounts of epinephrines do not exhibit the same potency as regards several physiologic responses(9). At the highest dose level of ergotamine tartrate used in these experiments, the presence of relatively large amounts of steroids might have overridden the blocking effect. When half the dose of ergotamine tartrate was used, a lesser degree of inhibition occurred even in the adrenalectomized animals.

Accumulation of fat in the liver due to the insult of ethionine administration appears to respond somewhat differently from peripheral depot fat metabolism with regard to blockade with ergotamine(5). Even in animals with intact adrenals, this blocking drug inhibited development of a fatty liver. It is at the liver site also that ergotamine had its most marked effect on glycogenolysis due to epinephrine. Our data suggest that the extra-hepatic effects of ergotamine on fat metabolism are masked by the presence of cortical steroids.

*Summary.* Ergotamine tartrate significantly inhibited movement of fat out of the epididymal fat body in adrenalectomized animals during a 12 hour fast. This effect was not observed in intact animals under similar circumstances. Neither hexamethonium chloride nor Dibenzyline affected fat mobilization in either intact or adrenalectomized animals. It is suggested that the adrenal steroids and the epinephrines may act together to regulate fat cell metabolism.

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# Fractionation of Sera from Guinea Pigs at Different Stages of *Brucella abortus* Infection.[*] (24446)

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Previous work showed that guinea pigs infected with *Brucella abortus* could not be reinfected while living organisms of the original infection were demonstrable in the tissues, then resistance waned until animals regained susceptibility(1). Subsequent investigations demonstrated that mononuclear phagocytic cells and humoral factors played an important role in resistance(2). Qualitative and quantitative alterations in serum protein components as a result of other infections have been demonstrated(3,4,5). Results obtained by electrophoretic fractionation of serum protein components during the course of experimental brucella infection are here reported.

*Materials and methods.* Male guinea pigs weighing 400 to 500 g and kept on diet of Purina rabbit feed, green grass and water were inoculated subcutaneously with 3 x $10^7$ *Brucella abortus* organisms (strain SAS). Infection was allowed to progress ½, 1, 2, 3, 4, 5, 8, 10 and 12 months. Animals were bled at end of each period and serum separated after overnight refrigeration. Total protein determinations were made by modification of Kingsley's method(6). Sera were fractionated at room temperature for 16 hrs at 5 milliamperes in Spinco Model R—Series D paper electrophoresis cell with Whatman 3 mm filter paper strips and barbital buffer pH 8.6, ionic strength 0.075. Strips were dried and stained by Durrum's method(7). Stained strips were scanned, ionograms plotted and areas under curves integrated with Spinco Analytrol.

*Results.* Five fractions appeared as shown in Fig. 1: Albumin, and alpha-1, alpha-2, beta and gamma globulins. All fractions, except gamma globulin, migrated towards the anode. Fifteen days after inoculation infection was well established as shown by abundant growth of brucella from the spleen, and positive agglutination titers of 1:50 to 1:100. At this stage of infection the electrophoretic pattern was the same for infected animals and uninfected controls (Fig. A). Guinea pigs infected for 1 and 2 months gave agglutinin titers of 1:200 and 1:400 respectively but again the electrophoretic patterns were not significantly different from the normal. Three to 4 months after infection, however, marked differences between uninfected controls and infected animals were evident (Fig. 1). Average agglutinin titer at this stage of infection was 1:1,600 and overall changes in the electrophoretic pattern may be attributable to an increase in gamma globulin. Five to 8 months after infection the average agglutinin titer was 1:800 and a decrease in the gamma globulin concentration was evident. Twelve months after infection the electrophoretic pattern closely approximated that of the uninfected controls and the agglutinin titer ranged from 1:160 to 1:400.

Quantitative data in terms of percentage composition and their conversion to concentration of protein components in terms of mg/ml offered a better evaluation of the changes observed during infection (Table I). Total protein in controls remained fairly constant throughout the experiment, averaging 62 mg/ml and relative concentration of all components exhibited little variation. In infected animals total protein increased from 61 mg/ml at 15 days to a high of 66 mg/ml at 3 months, then decreased to 56 mg/ml at 12 months. Gamma globulin concentration increased approximately 50% 3 to 4 months after infection, as compared to uninfected controls, and then returned to low levels. Albumin concentration fell from 23 mg/ml 15 days after infection to 16 mg/ml at 4 months. This decrease coincided with an increase of gamma globulin. At the end of 12 months albumin concentration was 17 mg/ml while no gamma globulin increase was apparent. This latter

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[*] These studies have been supported by USPHS grant.

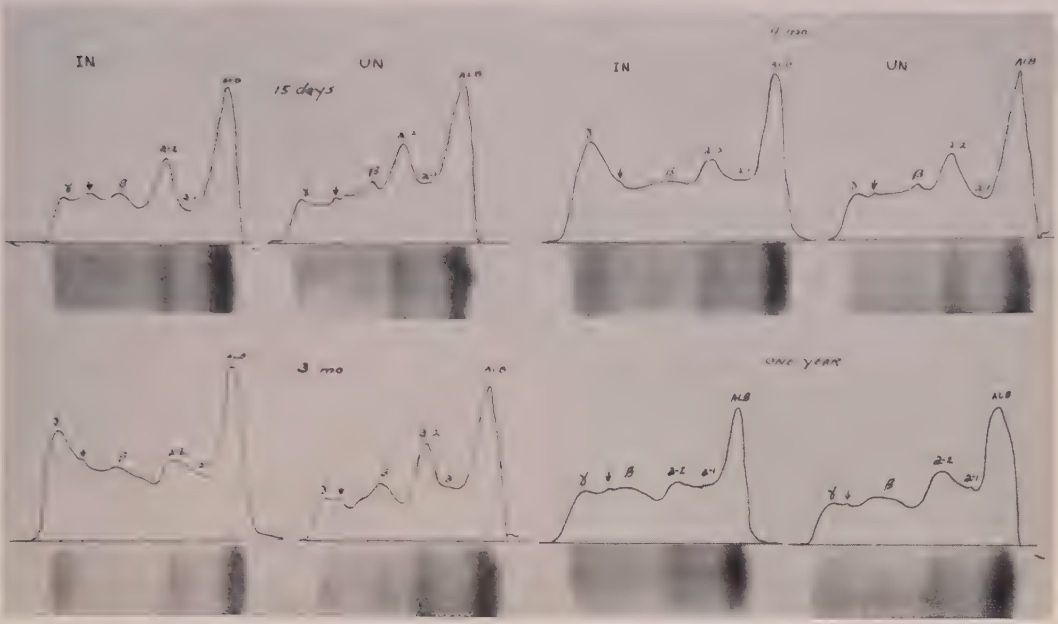

FIG. 1. Electrophoretic patterns of sera of uninfected and infected guinea pigs during different stages of experimental *Br. abortus* infection. Arrows indicate origin.

effect may be due to decrease in the total protein in infected animals at this stage of infection.

Increase in globulin with a decrease in albumin seems a characteristic serum alteration in many diseases. The fall in serum albumin has been thought to follow serum globulin increase and to depend upon a regulatory

TABLE I. Average Concentration and Percentage of Serum Protein Components in Infected and Uninfected Guinea Pig Sera.

| | | | Components | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Duration of infection, mo | | No. of animals | Albumin | | α-1 | | α-2 | | β | | γ | | Total protein, mg/ml |
| | | | Conc. | % | Conc. | % | Conc. | % | Conc. | % | Conc. | % | |
| ½ | IN | 4 | 23 | 38 | 3 | 4 | 13 | 21 | 8 | 14 | 14 | 23 | 61 |
| | UN | 4 | 23 | 37 | 3 | 6 | 13 | 21 | 10 | 16 | 12 | 20 | 61 |
| 1 | IN | 12 | 23 | 36 | 3 | 5 | 13 | 21 | 9 | 15 | 14 | 23 | 62 |
| | UN | 5 | 24 | 39 | 3 | 6 | 14 | 23 | 7 | 12 | 12 | 20 | 60 |
| 2 | IN | 4 | 21 | 34 | 4 | 6 | 13 | 21 | 10 | 16 | 14 | 23 | 62 |
| | UN | 4 | 23 | 37 | 5 | 8 | 16 | 25 | 7 | 11 | 12 | 19 | 63 |
| 3 | IN | 8 | 18 | 27 | 5 | 9 | 13 | 19 | 10 | 15 | 20 | 30 | 66 |
| | UN | 4 | 23 | 37 | 4 | 6 | 15 | 24 | 10 | 16 | 11 | 17 | 63 |
| 4 | IN | 5 | 16 | 26 | 4 | 6 | 11 | 18 | 6 | 9 | 26 | 41 | 63 |
| | UN | 4 | 22 | 37 | 2 | 3 | 15 | 26 | 8 | 13 | 13 | 21 | 60 |
| 5 | IN | 5 | 19 | 31 | 7 | 11 | 12 | 19 | 9 | 14 | 15 | 25 | 62 |
| | UN | 4 | 23 | 37 | 3 | 4 | 16 | 25 | 8 | 13 | 13 | 21 | 63 |
| 8 | IN | 4 | 19 | 30 | 4 | 7 | 15 | 24 | 10 | 16 | 14 | 23 | 62 |
| | UN | 4 | 23 | 36 | 4 | 7 | 14 | 22 | 10 | 16 | 12 | 19 | 63 |
| 10 | IN | 4 | 22 | 35 | 3 | 5 | 14 | 22 | 8 | 13 | 15 | 25 | 62 |
| | UN | 3 | 22 | 36 | 4 | 6 | 11 | 18 | 10 | 17 | 14 | 23 | 61 |
| 12 | IN | 4 | 17 | 30 | 6 | 11 | 12 | 21 | 8 | 15 | 13 | 23 | 56 |
| | UN | 3 | 21 | 35 | 4 | 7 | 14 | 23 | 9 | 15 | 12 | 20 | 61 |

Stand. dev. for protein components from uninfected sera is 0.9 to 1.

mechanism to keep osmotic pressure within certain limits(8). The rise in globulin has been attributed to (a) antibody formation, (b) alteration of relative rates of production and utilization of albumin and globulin, and (c) traumatic shock(8,9,10). Alpha-1 and alpha-2 globulin concentration did not vary more than 3-4 mg/ml throughout the experiment. Changes in concentration for both alpha-globulins occurred within the 4-5 month infection period (Table I). The beta-globulin fraction exhibited very little change. Lack of variation in this fraction at 3-4 months, when significant alterations were taking place, could be due to the fact that as the gamma-globulin rose in concentration it tended to mask the increase, if any, of the beta-fraction in electrophoretic patterns.

*Summary and conclusions.* 1. New serum components were not detected in infected animals. 2. Significant increases in gamma-globulin fraction were observed during 3-4 month infection period. 3. The rise observed in gamma-globulin coincided with highest agglutinin titers in sera of infected animals during 12 months experimental period. 4. Absorption studies are being carried out in an effort to determine if the gamma-globulin rise is due mainly to antibody formation.

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## Influence of Urine Volume on Output of Corticoids by Normal Human Subjects.* (24447)

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It has been noticed that the output of adrenal cortical steroids appeared to be dependent upon urine volume. This does not imply that the cortex was activated by changes in water balance, but rather that alteration in filtration and/or reabsorption in the kidney with urine flow influenced steroid output. Lloyd (1) has presented data to demonstrate this relationship in Addisonian and normal patients. Marks and Leaf(2), Kalant(3) and Lazo-Wasem *et al.*(4) have confirmed the results in dog, rat, and guinea pig. This is a report of the influence of urine volume on urinary steroid output in normal subjects under various conditions of salt and water load. As a corollary, renal clearances of steroids were also determined.

*Methods.* Our tests were performed on 24 normal medical students. Three different procedures were adopted to provide short and long term evaluation of the problem. 1. *Long term study (day by day).* Students on a relatively constant diet and in good health, collected 24 hour urine specimens kept in the cold and preserved with small amount of glacial acetic acid. The subjects voluntarily restricted water to a minimum, or drank water to excess, or maintained a normal water intake throughout the 24 hours. In another series of tests the subjects took 40 mg of hydrocortisone/day in a divided dose while controlling water intake. Each sample was

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TABLE I. Excretion of Total Corticoids in 24-Hour Urine Sample in Relation to Volume of Urine with and without Added Cortisone. A recovery period of 10 days occurred between each phase of the tests.

| Condition of subject | No. of tests | Urine vol, ml/day | Steroid excreted, mg/day |
|---|---|---|---|
| Water deprivation | 5 | 773 ± 80* | 8.1 ± 1.2* |
| Water intake: | | | |
| Normal | 8 | 1270 ± 130 | 9.3 ± 2.4 |
| High | 9 | 2430 ± 360 | 12.7 ± 2.2 |
| Intake (40 mg cortisone): | | | |
| Low | 3 | 750 ± 210 | 14.0 ± 2.8 |
| Normal | 3 | 1400 ± 360 | 34.0 ± 4.6 |
| High | 3 | 2130 ± 93 | 45.0 ± 1.2 |

* Stand. dev.

assayed for corticoids by the method of Reddy as modified by Brown(5). The method determines conjugated and free 17-hydroxycorticoids which represent about 85% of the daily adrenal output. 2. *Short term study*. Control levels of urinary steroids were determined after which each man rapidly drank 1 l of 1.4% NaCl, 0.9% NaCl, 0.54% NaCl, or tap water. Urine samples were collected at successive 20-minute intervals for the following 2 hours. Other subjects were given water to drink and after excretion of water reached high levels (about 40 minutes) 2 units of ADH (Pitressin, Parke-Davis) were given in the muscle. Urine samples were collected at 20-minute intervals. All samples were analyzed for steroid as outlined above. 3. *Renal clearances of corticoids*. Urine was collected over a 30-minute period in well hydrated subjects with good urine flow rates. Heparinized blood samples were taken at midpoint of each period. Urine and plasma were extracted with chloroform to obtain "free" steroids and with butanol to obtain "bound"† corticoids. The method of Reddy(7) was used to precipitate proteins in plasma before extraction. Duplicate portions of plasma and urine samples were analyzed for creatinine to determine the glomerular filtration rate (GFR). The method of Hare(6) was used to obtain preformed creatinine and standard picric acid method was utilized for color development.

*Results*. Definite correlation was found between urine volumes and adrenal corticcal steroid output in urine (Table I). Although differences in steroid excretion between low and normal water intake and between normal and high water intake were not significant, the trend was obvious and the difference between low and high water intake figures were highly significant.

A similar relationship could be shown (Table II) on a short time basis. An increase in urine output resulted in increased adrenal steroid output in urine regardless of salt intake. In those cases in which the change in

TABLE II. Relationship between Urine Volume and Steroid Output in Normal Subjects Drinking One Liter of Indicated Solution. Water excretion expressed as ml/20 min. period and steroid as µg/20 min. period. Each value represents average of 5 subjects.

| | Water | | .54% NaCl | | .90% NaCl | | 1.40% NaCl | | Water & ADH | |
|---|---|---|---|---|---|---|---|---|---|---|
| Period | Urine vol | Steroid output | Urine vol | Steroid output | Urine vol | Steroid output | Urine vol | Steroid output | Urine vol | Steroid output |
| 1* | 18 | 92 | 16 | 171 | 23 | 113 | 91 | 109 | 12 | 123 |
| | | | All fluids taken after first control period | | | | | | | |
| 2 | 70 | 168 | 31 | 236 | 44 | 97 | 44 | 106 | 34 | 146 |
| 3 | 198 | 186 | 54 | 291 | 46 | 75 | 24 | 127 | 106 | 302 |
| | | | | | | | | | ADH given | |
| 4 | 164 | 180 | 19 | 196 | 34 | 83 | 18 | 124 | 32 | 95 |
| 5 | 66 | 143 | 13 | 152 | 21 | 78 | 19 | 114 | 10 | 81 |
| 6 | 18 | 88 | 15 | 160 | 20 | 52 | 14 | 100 | 13 | 119 |

* Each successive period is consecutive 20 min. period with no time delay between periods.

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† In urine the steroid is "bound" largely to glucuronide and may be liberated by enzymatic hydrolysis. The exact binding in plasma is not clear but presumably may be to protein as well as to glucuronide.

TABLE III. Plasma and Urinary Levels of Conjugated and Free Steroids and Clearances of These Fractions in Normal Subjects. Urine collections were made over 30 min. periods with blood samples at the mid-point. Clearances are expressed in the usual way: $UV/P = C$.

| | | Free steroid | | | Conjugated steroid | | |
|---|---|---|---|---|---|---|---|
| Subject | Creatinine clearance, ml/min. | Urine, μg/ml | Plasma, μg/% | Clearance, ml/min. | Urine, μg/ml | Plasma, μg/% | Clearance, ml/min. |
| APR | 92 | .72 | 118 | .01 | 7.5 | 168 | 4.78 |
| HRB | 71 | .99 | 46 | 1.1 | 10.8 | 118 | 5.18 |
| | 82 | 1.0 | 46 | 1.8 | 9.5 | 110 | 7.00 |
| EWH | 80 | .7 | 30 | 1.5 | 1.56 | 64 | 1.6 |
| | 83 | 1.6 | 100 | 1.9 | 2.8 | 160 | 2.1 |
| EHE | 86 | .2 | 23 | 1.2 | 3.8 | 65 | 6.1 |
| EHE* | 96 | 6.3 | 350 | 9.8 | | | |
| GHR | 82 | | | | 4.8 | 118 | 4.70 |
| AVA | 77 | | | | 8.6 | 288 | 4.10 |

* 15 min. after infusion of 50 mg hydrocortisone.

urine output was not so marked as with 0.54% NaCl, changes in steroid excretion were also not marked. The greatest change was in subjects given ADH. Large water volumes and corticoid excretions were obtained upon hydration. When ADH was administered, a sudden drop in excretion of both corticosteroids and water occurred within a 20-minute period. It would be impossible to state whether any alteration in corticoid excretion *per se* is due to ADH acting directly on the adrenal gland rather than the kidney. Some workers have suggested that ADH may activate the adrenal, but this possibility would appear unlikely for all urine samples were analyzed for sodium with the flame photometer and total excretion per period did not show significant variations.

As a corollary of the corticoid excretion studies reported above, renal clearances were determined in normal subjects. Clearance of creatinine was used to measure glomerular filtration rate. Excretions of adrenal steroids in urine were similar to those reported in Table II; plasma level of "bound" corticoids averaged 58 to 128 μg % and "free" steroid averaged 13 to 46 μg %. The plasma ratio of free/bound corticoids was about 1:4. These values are similar to those reported by Reddy(7). Urinary ratios have been previously reported by Brown(5). Renal clearance data are presented in Table III. The clearances were very consistent with clearance of "bound steroid" higher in all cases (averaging about 4 ml/min.). These values indicate a greater reabsorption of the free molecule and a greater excretion of the bound form which would be predicted. Both forms must be reabsorbed to a considerable extent. The usual ratio of free/bound corticoids in urine is about 1:8, which confirms the greater excretion of the bound form. With a high degree of reabsorption by the kidney tubule, it is not surprising that great variation in urine flows would produce variation in amount reabsorbed and therefore in excretion of steroids.

These data indicate that there is a considerable influence of urine volume upon excretion of steroid, and point up the fact that this must be taken into account in evaluation of the steroid output in patients with great variation in urine volume.

*Summary.* The influence of urine volume upon excretion of corticoids has been determined in normal human subjects. There was a direct correlation with increases in urine volume resulting in an increase in steroid output whether measurement was made on a long time basis (day by day), or a short time basis (minute by minute). Subjects tested under waterloads and salt loads and with ADH also demonstrated the same positive correlation. As a corollary, clearances of steroids were measured and found to be about 1 ml/min. for free corticoids and about 4 ml/min. for bound steroids.

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## Partial Purification and Properties of Lupus Erythematosus Cell Promoting Factor. (24448)

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Patients with disseminated lupus erythematosus (DLE) have a substance in their serum which is thought responsible for formation of the characteristic "lupus erythematosus cells." Previous attempts at isolation of this substance have indicated that it is associated with the gamma globulin fraction (1). A useful method for chromatographic fractionation of proteins using cellulose exchangers has recently been developed(2). The technic is carried out under conditions which favor preservation of the structure and biologic activity of the proteins recovered in the fractions. The wide range of applicability of this method is reflected in the reports appearing in the literature(3). A previously reported preliminary study(4) indicated that an anionic cellulose exchanger may effect some separation of the L-E factor. The purpose of this communication is to report on some of the properties of a preparation containing L-E activity by use of a cationic cellulose exchanger.

*Methods.* Sera obtained from normal individuals and from patients with DLE were stored in frozen state until ready for use. All fractionation procedures were carried out in cold room at 4°C. Isolation of serum gamma globulins was done by electrophoretic convection in 0.15M phosphate buffer at pH 7.4. Nitrogen determinations were done by micro-Kjeldahl technic and phosphorus values were obtained by Schmidt-Thannhauser technic (5). The resulting products contained little or no contaminating beta globulin as determined by moving boundary and paper electrophoresis. However, when the products were examined by agar diffusion(6) using rabbit anti-human gamma globulin as the developing agent, as many as 7 distinct antigenic components could be identified. The gamma globulin isolated from patients with DLE gave strongly positive reactions in the L-E cell preparations(7) and in the DNA-protein coated latex agglutination test(8). Serum samples and gamma globulin fractions from normals and subjects with DLE were prepared for column chromatography by preliminary dialysis against 0.05M phosphate buffer at pH 5.0. A small amount of insoluble material, usually present at end of 24 hours, was removed by centrifugation. When whole serum was to be chromatographed, the dialyzed sample was diluted 1:10 with the starting buffer before placing it on the column. Gamma globulin samples were placed on the column without preliminary dilution. Approximately 15 g of cationic exchange cellulose, CM-cellulose-SF,† were placed in 3 changes of 0.05M phosphate buffer at pH 5 for 48 hours. The slurry was placed in a 20 x 200 mm glass column and packed until a standard height with a flat crown had been achieved. Under these conditions, the flow rate varied from 60 to 80 ml/hour. The pH and salt concentration of the eluting buffers was increased simultaneously. The initial pH was 5 and final pH 7; initial salt concentration was 0.05M and final concentration 0.15M. Salt concentration was increased by step-wise addition of sodium chloride. Six ml samples

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† Brown & Co., Berlin, N.H.

TABLE I.

| | L-E $\gamma$ globulin | Positive column fraction |
|---|---|---|
| L-E cells | Yes | Yes |
| Agglutination | ” | ” |
| Total N | 100 mg | 2.8 mg |
| N conc. | 1.83 mg/ml | .428 mg/ml |
| Phosphorus conc. | .042 ” | .030 ” |
| N/P ratio | 44 | 14.3 |

were recovered with an automatic fraction collector operating in room at 4°C, at flow rate of 60 to 70 ml/hour. Elution curves were constructed plotting fraction number against optical density at 280 mU. Selected groups of consecutive fractions showing heavy absorption were pooled and concentrated against 0.15M sodium chloride by negative pressure dialysis. The resulting concentrates were characterized as follows: L-E cell promoting activity; DNA-protein coated latex agglutinating activity; nitrogen and phosphorus content; electrophoretic and ultracentrifugal properties; immunologic behavior in gel diffusion studies.

*Results.* Elution diagrams of normal serum and serum from patients with DLE are qualitatively similar. As indicated, evidence of L-E activity was present in one of the concentrated pooled fractions. Only rosettes were seen in the L-E cell preparation but this material resulted in a strongly positive DNA-protein coated latex agglutination test.

Fig. 1 presents typical elution diagrams of gamma globulin fractions prepared from normal individuals and from patients with DLE.

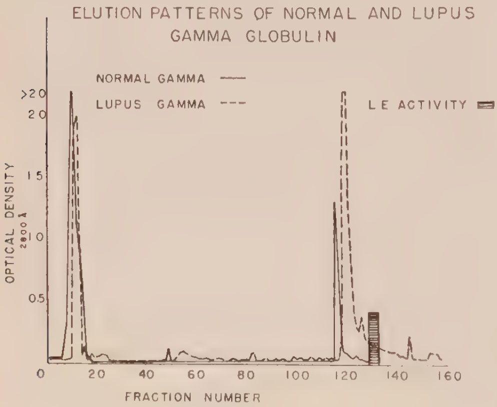

FIG. 1.

The relatively minor differences in the patterns are of questionable significance since they could result from variations in column packing, column size, buffer flow rates, and rates of change in pH and salt concentration. A pooled concentrated fraction could be recovered from L-E gamma globulin which demonstrated L-E activity in both agglutination test and in L-E cell test (Fig. 2). The positive fractions were regularly obtained at a pH between 6.8 and 7.0 and at a salt concentration of 0.15M.

In a typical experiment, 100 mg of L-E gamma globulin nitrogen was placed on the column. L-E activity was recovered in a pooled concentrated fraction with approximately the same activity intensity as the starting material. In Table I, data are shown comparing the properties of the starting material with those of the positive fraction. Activity could be demonstrated in a preparation which had lost 97% of its original total protein nitrogen. Of some interest is the reduction in nitrogen to phosphorus ratio from 44 to 14.3. Since the first major elution peak had a nitrogen to phosphorus ratio of 13, it is presumed that a considerable amount of gamma globulin remained fixed on the column.

The positive pooled fraction was examined in the Tiselius moving boundary electrophoresis apparatus and with conventional paper electrophoresis. A single peak with the mobility of normal gamma globulin was observed. When placed in the ultracentrifuge, the fraction showed a single peak with a sedimentation constant of 7 Svedberg units.

The starting L-E gamma globulin preparation regularly showed as many as 7 distinct antigenic components when examined by the Ochterlony technic; the positive fraction, however, failed to demonstrate more than a single band. In addition, this single band showed a reaction of identity with a component of normal gamma globulin. No band was observed when the positive fraction was set up in the agar diffusion against purified DNA over a pH range of 7.4 to 8.2. The gel diffusion properties of the "leuco-precipitines" described by Seligman are similar to our findings except we were unable to demon-

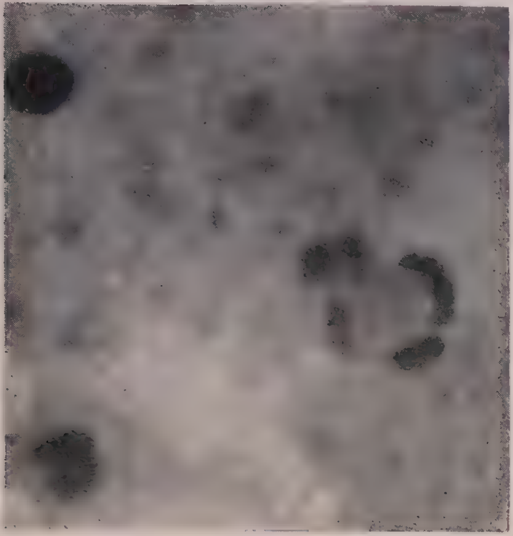

FIG. 2.

strate a precipitin reaction between the L-E factor and DNA(9).

The first major peak, shown in the elution pattern of L-E gamma globulin in Fig. 1, regularly showed from 3 to 5 components when examined by the Ochterlony technic. On 2 occasions, fractions in the first peak demonstrated a positive L-E agglutination test but none of the fractions from this peak produced a positive L-E cell preparation.

*Discussion.* This study illustrates another use of cellulose exchangers in chromatographic separation of serum proteins. The product showing L-E activity exhibited electrophoretic and sedimentation properties similar to products prepared by other methods(10). Within the limitations of the technics employed, the L-E factor appears to behave as a single component. The data do not support the position that more than one specific factor present in L-E sera is responsible for hematoxylin body formation. The data do suggest that the L-E factor is a substance associated with gamma globulin which shares antigenic groupings with normally occurring gamma globulin. This view receives further support from the observation in our laboratory that rabbit anti-normal human gamma globulin serum is capable of removing the L-E activity from positive preparations (to be published). In addition, fluorochrome studies (11) indicate that, when the L-E factor and nucleoprotein become associated, normal gamma globulin is added to the complex.

The suggestion is often advanced that the L-E factor is an "antibody" to a nuclear component but the evidence available does not permit this conclusion. The amount of protein nitrogen necessary to retain full biologic activity of the L-E phenomenon is small. The unusual sensitivity of the L-E phenomenon to pH, temperature, storage and dilution, and the sequence of events as seen under phase microscopy(12), are not reminiscent of an ordinary antigen-antibody reaction. An alternative suggestion is that L-E activity may be conferred on the normally occurring gamma globulin by its association with a small but highly active abnormal component.

*Summary.* Partial purification of the L-E cell promoting factor from gamma globulin has been achieved with the aid of a cationic cellulose exchanger. The fraction containing L-E activity is eluted at a pH of approximately 7 and a salt concentration of 0.15M. In the process of purification, total protein nitrogen content was reduced 97% nitrogen to phosphorus ratio was reduced from 44 to 14.3 without notable reduction in biologic activity. This fraction possesses the electrophoretic, sedimentation and immunologic properties of a normal gamma globulin.

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# Red Cell and Plasma Transit Through Spleen: the Splenic Hematocrit.* (24449)

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Considerable interest in the physiology of the spleen has been shown since Barcroft and co-workers(1-6) showed its importance as a reservoir for red blood cells. This function has been confirmed by Haam(7) and Kramer (8). Kramer concluded on effect of hypoxia on systemic hemoglobin content and spleen weight, that the spleen consists almost entirely of packed red cells. Reeve(9) postulated from $P^{32}$ blood volume determinations that the spleen concentrates red blood cells. Everell(10) calculated splenic hematocrit in rats, after allowing 15 minutes for red cell mixing with injected labeled red cells. He cound the hematocrit to be 46.9 in female rats and 50.7 in the males. These values would raise a question when compared to Kramer's(8) conclusion that the spleen consists almost entirely of packed red cells. However, Barcroft(1) using CO, had previously demonstrated that 30 to 90 minutes elapses before the general circulation and general mass of hemoglobin in the spleen acquire the same CO content. This indicates that perhaps Everell did not allow sufficient time for red cell mixing to occur. The following experiments were designed to determine the time necessary for equilibrium to be reached between general systemic circulation and splenic venous outflow. This enabled calculation of mean transit time of plasma and red blood cells and provided data from which the splenic hematocrit could be determined.

*Methods.* Eight healthy mongrel dogs weighing 9 to 12 kg were anesthetized with sodium pentobarbital, 25 mg/kg. A generous midline abdominal incision was made and the spleen exposed. One of the large splenic veins was dissected free, care being taken that adequate collateral venous drainage was left intact. This vein was incised and a polyethylene catheter tied into the proximal segment. The free end of the catheter contained a stopcock. Similar polyethylene catheters were inserted into the femoral artery and femoral vein. In 6 experiments, aortic pressure was monitored with a mercury manometer connected to the femoral arterial catheter. Twelve ml of a saline suspension of the dogs' red cells previously tagged with approximately 500 microcuries of $Cr^{51}$ and washed free of any extracorpuscular $Cr^{51}$ activity was mixed with 125 microcuries of $I^{131}$ tagged human serum albumin. In one experiment, $I^{131}$ gamma globulin was used instead of $I^{131}$ serum albumin. This mixture was then injected over an 8 to 10 second period into the femoral vein. From the start of injection until 5 minutes later, femoral arterial blood and splenic venous blood was collected continuously at 30 second intervals into paraf-

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† Medical Student Fellow, Nat. Foundation for Infantile Paralysis.

‡ Established Investigator, American Heart Association.

TABLE I. Splenic Red Cell and Albumin Transit Times in 8 Dogs.

| Arterial B.P. (mg/Hg) |  | Equil. time (min.) |  | Mean transit time (min.) |  |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|
| Zero time | Equil. time | Red cell | Plasma | Red cell $T_r$ | Plasma $T_p$ | $T_r/T_p$ | Hct. l.v.† | Hct. spl. | Hct. spl. / Hct. l.v. |
|  |  | 87.5 | 32.5 | 39.82 | 5.29 | 7.53 | 49.5 | 88 | 1.8 |
|  |  | * 87.5 | 15.0 | 34.03 | 8.28 | 4.1 | 31 | 65 | 2.1 |
| 140 | 118 | 26.0 | 3.8 | 6.8 | .72 | 9.44 | 46 | 89 | 1.9 |
| 128 | 100 | 56.0 | 4.0 | 11.16 | 1.01 | 11.04 | 42 | 89 | 2.1 |
| 108 | 96 | 62.5 | 15.0 | 18.54 | 4.41 | 4.20 | 38 | 72 | 1.9 |
| 114 | 100 | 47.5 | 27.5 | 9.41 | 2.43 | 3.87 | 37 | 70 | 1.9 |
| 140 | 61 | 85.0 | 31.0 | 26.4 | 6.90 | 3.82 | 36 | 68 | 1.9 |
| 125 | 75 | 22.5 | 3.5 | 6.52 | .98 | 6.65 | 38 | 80 | 2.1 |
| Avg |  | 59.3 | 16.5 | 19.09 | 3.75 | 6.33 | 39.7 | 77.6 | 1.96 ± .12‡ |

* $I^{131}$ gamma globulin used instead of $I^{131}$ serum albumin.

† Hct. l.v. was determined by centrifugation of arterial blood.

‡ ± stand. dev.

fined test tubes containing dried heparin solution. Intermittent samples were then collected every 5 minutes until 30 minutes had elapsed and thereafter every 10 minutes until 2 hours had elapsed from the time of injection. (Only experiments in which splenic venous catheter blood flow remained constant during the collection period were utilized in this study.) Following collection of the samples, 0.5 ml aliquots of whole blood were pipetted into glass vials. These were centrifuged and the red blood cells washed 3 times with normal saline, care being taken not to remove any of the red cells. The $Cr^{51}$ activity of these washed aliquots were counted in a well-type scintillation counter, and the activity expressed as $Cr^{51}$ counts per ml whole blood. The remaining blood in the collection tubes was centrifuged and 0.3 ml aliquots of the supernatant plasma were counted in a similar manner, the activity being expressed as $I^{131}$ counts per ml plasma. From the $Cr^{51}$ counts per ml in each sample and its time of collection (corrected for collecting system catheter delay and using the midpoint of the injection period as zero time), a red cell concentration vs. time curve was constructed for arterial and splenic venous blood. From the plotted curve, equilibrium time and equilibrium concentration were determined. From this data the red cell mean transit time was calculated employing a formula modified from the method of Bradley and co-workers(11) for estimating splanchnic blood volume. Mean transit time was taken as equal to the difference between mean arterial and venous concentrations from zero time to equilibrium time divided by equilibrium concentration. Similarly, plasma albumin mean circulation time was determined from the arterial and venous $I^{131}$ counts. The hematocrit of the spleen was calculated directly from the ratio of mean transit times and large vessel hematocrit employing the formula previously derived by Lilienfield and co-workers(12,13).

*Results.* The time for red cell equilibrium to be reached between the splenic vein and femoral artery was longer in all experiments than the time for plasma equilibrium to occur. Average time for red cell equilibrium was 59.3 minutes (22.5 to 87.5). Average time for plasma equilibrium was 16.5 minutes (3.5 to 32.5). Red cell mean transit time as calculated by the above formula was 19.1 minutes (6.8 to 39.8). Plasma albumin mean transit time was 3.8 minutes (0.7 to 8.3).

Average hematocrit of the spleen was calculated as 77.6 (65 to 88). Ratio of splenic hematocrit to large vessel hematocrit averaged 1.96 ± .12 (S.D.).

*Discussion.* Sodium pentobarbital was chosen as the anesthetic agent because it causes relaxation of the spleen(14). It was thought that it would prevent the reflex contraction induced by the trauma associated with catheterizing the splenic vein and more closely simulate the physiological status of the spleen in the intact animal. For this same reason, care was taken to insure adequate splenic venous drainage when the splenic vein was catheterized.

The method of determining splenic hematocrit from transit time data requires that the blood flow remain constant. In 4 of the experiments the arterial pressure fell from 125 mm Hg to 105 mm Hg. This fall was considered insignificant so that a constant blood flow during this period could be assumed.

However, in 2 experiments, the fall was from 130 mm Hg to 70 mm Hg at the time of red cell equilibrium. The results obtained from these experiments did not significantly differ from the others, therefore, the data were considered valid. It appears that the splenic blood flow remains rather constant over a wide range of blood pressure variations in a pentobarbital anesthetized dog.

The wide range of times (22.5 to 87.8 minutes) required for red cell equilibrium to occur agrees well with Barcroft's(1) determinations that 30 to 90 minutes are required for red cell equilibrium in the spleen. This would indicate that a longer time than the usual 20 minutes should be used when determining blood volumes in dogs with $Cr^{51}$ tagged red blood cells. It would probably be best to use the upper limit of 90 minutes to insure adequate time for red cell mixing. The low splenic hematocrit figures obtained by Everell(9) were most likely the result of not permitting sufficient time for equilibrium.

It is notable that in this study the splenic hematocrit was very nearly twice the large vessel hematocrit (average 1.96 ± .12 S.D.) over a range of arterial hematocrits from 31 to 49.5. This supports the contention that the dog spleen contains red cell concentrated blood.

*Summary.* Five-hundred microcuries $Cr^{51}$ tagged red blood cells and 125 microcuries $I^{131}$ tagged serum albumin was injected into 8 sodium pentobarbital anesthetized dogs and blood samples collected simultaneously from the femoral artery and splenic vein. Time for red cell equilibrium was 59.3 minutes (22.5 to 87.5 minutes). Plasma equilibrium time was 16.5 minutes (3.5 to 32.5 minutes); red cell mean transit time was 10.09 minutes (6.8 to 39.82 minutes). Plasma mean transit time was 3.75 minutes (0.72 to 8.28 minutes). Average hematocrit of the spleen was 77.6 (65 to 88). Ratio of splenic hematocrit to large vessel hematocrit was 1.96 ± .12 S.D. over a range of arterial hematocrits from 31 to 49.5.

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# Alteration of Homograft Reaction by A-methopterin in Lethally Irradiated Mice Treated with Homologous Marrow. (24450)

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Mice exposed to lethal total-body X irradiation which receive an intravenous inoculation of homologous marrow may survive acute irradiation damage. Later, they may succumb to a homograft reaction. Although it is generally accepted that there is an immune response, the problem of whether this reaction is initiated by host(1) or graft(2) has not been resolved. Methods of precluding this homograft reaction have been studied with little success(3). Increased long-term survival(4,5) may be readily obtained following use of embryonic hematopoietic tissue as a protective agent in place of adult marrow cells. This procedure is limited in usefulness since complete absence of homograft reaction (2) is restricted to certain strain combinations (unpublished data). Drug treatments as a method of modifying the homograft reaction have proved generally unfavorable(3). An attempt was made to apply the observations of V. H. Haas and M. Potter (personal communication), on inhibitory effect of A-methopterin on immune response in mice, to the problem of preclusion of homograft reaction in lethally irradiated mice treated with homologous marrow. This preliminary report shows alteration in homograft reaction in male and female mice by administration of folic acid antagonist, A-methopterin.* This alteration consists of an increase in survival in several groups and absence of a reaction in 2 groups.

*Materials and methods.* At 3 to 4 months of age mice were exposed to 800 r total-body X irradiation. This is a 30-day LD 98 dose for $F_1$ hybrid mice. Two X-ray tubes placed opposite to each other were operated at 200 kvp and 15 ma.; filtration was 0.25 mm cu. and 0.55 mm Al with 54 cm from each focal spot to center of mouse, with dose rate of 120 r/min. Previously described experimental procedures for marrow preparation and treatment were followed(6). Male and female mice from inbred strains or $F_1$ hybrids between 2 inbred strains were used in host-donor combinations which had elicited an early severe reaction under these experimental conditions. A-methopterin was administered by intraperitoneal injection at 2 dose levels: 3 mg/kg body weight and 1.5 mg/kg body weight at 48 hour intervals for 5, 6 and 9 treatments. Drug treatment was started at 14 days following irradiation and homologous marrow inoculation. At this time the animals survived acute irradiation damage, body weight is returning to preirradiation level and homograft reaction has not become apparent. The variation in drug treatment was an attempt to acquire maximum beneficial effects, with minimum drug toxicity in lethally irradiated animals.

*Results.* A group of 23 (C57BL × DBA/2)$F_1$ hybrid male mice were exposed to 800 r total-body X irradiation. Five animals served as irradiated controls and were all dead by 12 days. The 5 animals which received isologous marrow and the 13 animals which received parental-strain C57BL marrow all survived acute irradiation damage. Of the 13 mice which received C57BL marrow, 7 animals were treated with intraperitoneal injections of A-methopterin at a dose of 1.5 mg/kg body weight at 48-hour intervals for 9 treatments and 6 animals served as controls. Fig. 1 shows curves for body weights recorded every 48 hours for these 3 groups of mice starting at 14 days post-irradiation.

In this graft-host combination there is an early, severe reaction. Although this reaction appears to be uniformly severe in all mice treated, 11% of the treated mice survived the reaction(2). In the group which received only homologous marrow treatment, one animal survived the reaction and weights for this mouse are represented by the broken-line curve. The weight of this animal declined

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* A-methopterin (Methotrexate) kindly supplied by Lederle Labs., Amer. Cyanamid Co., Pearl River, N. Y.

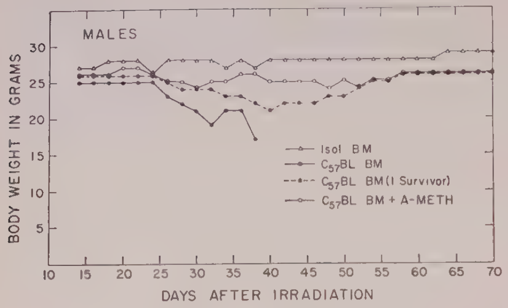

FIG. 1. Mean body wt of (C57BL × DBA/2)$F_1$ hybrid male mice receiving 800 r total body irradiation followed by isologous marrow inoculation, or parental-strain marrow, or parental-strain marrow and A-methopterin at a dose of 1.5 mg/kg × 9 q 48 hr. Wt curves start at 14 days post-irradiation when drug was first administered and continued until all mice were dead or recovery was established.

between 26th and 40th day representing the period when the reaction was grossly quite apparent. Eighteen days after lowest weight was recorded, this animal had fully recovered its weight loss. Average weights were recorded for all mice which did not survive the reaction and show a declining weight after 24 days until all animals were dead at 38 days.

In these hybrid male mice drug toxicity was noted near the end of series of drug treatments but there was quick recovery in most mice following suspension of treatment. One animal died on third day after drug was suspended and 2 animals failed to recover and died on days 50 and 56 following irradiation. These deaths were considered due to drug toxicity since the gross appearance of these animals was not that of a "typical" reaction in animals treated with homologous marrow following lethal irradiation. These animals appeared anemic, had enlarged spleens, and lymphoid tissue did not appear depleted as in animals dying of homograft reaction. The animals which survived drug treatment showed no secondary weight loss which might indicate a delayed homograft reaction. At 60 days post-irradiation these animals were grossly indistinguishable from those receiving isologous marrow, having already acquired the gray coat typical of black mice receiving high doses of irradiation. This group represents a 57% survival at 70 days.

While attempting to determine the dose of A-methopterin that would produce maximum beneficial effects and minimum toxic effects in these irradiated mice, several factors became quite apparent. Heavily irradiated animals do not tolerate A-methopterin as well as non-irradiated animals. One important factor may be the very short recovery period after irradiation and marrow treatment, as these animals barely regain their pre-irradiation weight when drug treatment is started. The previously observed(7) sex difference in toxicity to antifolic analogues was present, with females more sensitive than males to this drug treatment. It appears that to obtain maximum beneficial effects in suppressing the homograft reaction there must be some degree of toxicity. For example: In treating 10 (C57BL x DBA/2)$F_1$ hybrid males and 5 hybrid females with 3 mg/kg body weight of A-methopterin at 48-hour intervals for 5 treatments, females showed marked drug toxicity from which 4 of 5 mice recovered with no homograft reaction noted. On the other hand, 10 males showed no ill effects of this limited drug treatment, but 10 days following suspension of drug treatment, the beginning of a homograft reaction was noted. This reaction appeared quite "typical" with severe diarrhea, hunched appearance and ruffled coat. Although it appeared to be a severe reaction, 5 of these males recovered. It is not clear how much of this effect can be attributed to sex difference and how much to drug toxicity. It would appear that degree of depression of the reticuloendothelial system, as measured by toxicity, is important. There was toxicity noted and no discernible reaction (Fig. 1) at a total dose of drug less than in mice in which there was no toxicity but a reaction occurred, presumably because of insufficient drug treatment. The females which survived toxic effects of drug and the males which survived the reaction were still alive over 90 days post-irradiation.

In an attempt to reduce toxic effects of drug in female mice, 8 (C57BL x DBA/2)$F_1$ hybrid females were given A-methopterin at a dose of 1.5 mg/kg body weight at 48-hour intervals for 6 treatments. Drug toxicity was noted in these mice and weight loss occurred in the group receiving drug before it was observed in animals which received only homologous marrow. Although a severe homograft reaction was observed in drug-treated mice, all treated mice were still alive at 90 days after treatment. This reduced drug dose appears to be insufficient to suppress immune response in females although 9 treatments of the same dose proved effective in the male. CBA female mice irradiated, treated with C57BL marrow and given the modified drug treatment of 1.5 mg/kg body weight for 6 treatments gave results similar to those of the (C57BL x DBA/2)$F_1$ hybrid females.

Preliminary data are available on another hybrid combination, namely (C57BL x A)$F_1$ hybrid mice treated with C57BL marrow. In this case, the onset of reaction is later than in (C57BL x DBA/2)$F_1$ hybrids treated with C57BL marrow(2). When (C57BL x A)$F_1$ hybrid males were treated with 3 mg/kg body weight every 48 hours for 5 treatments starting at 14-day post-irradiation, drug treatment appeared deleterious. Although this observation must be confirmed, it would appear that drug treatment might lose its beneficial effect if administered too long before the expected onset of homograft reaction. Support for this belief is found in the work of R. Schwartz(8), who observed suppression of immune response in rabbits to bovine serum albumin as long as 6-mercaptopurine was administered. Upon suspension of drug treatment, the rabbits regained their ability to react against the foreign antigen. In the case of homograft reaction in irradiated mice treated with homologous marrow, it appears possible to permanently suppress this reaction as illustrated by male mice in Fig. 1 and female mice which received A-methopterin at 3 mg/kg body weight for 5 treatments at 48-hour intervals, or to reduce severity of the reaction as illustrated by increase in survival following the reaction in the other cases cited, if drug is administered during the time when the reaction would be apparent.

The mechanism of the action of A-methopterin in suppressing the homograft reaction in irradiated mice is obscure. This action appears to be of a fundamental nature since it is also possible to increase survival time of skin grafts by a similar treatment (unpublished data).

Although this is a preliminary report and further work is necessary, the results indicate that this may well be a profitable approach to the problem of preclusion of homograft reaction in irradiated mice treated with homologous marrow as well as other types of homograft reactions.

*Summary.* Mice which would normally succumb to a homograft reaction following lethal, total-body X irradiation and homologous marrow inoculation, may be spared if they are treated with folic acid antagonist, A-methopterin. Using different treatment schedules, varying degrees of effectiveness from increased survival following a typical reaction, to no detectable reaction, may be obtained. The relationship between drug toxicity and effectiveness in altering the homograft reaction is discussed.

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# Chronic Subclinical Infection with Mouse Salivary Gland Virus. (24451)

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Studies conducted by Rowe *et al.*(1) demonstrated persistence of human salivary gland virus in some children for at least 15-24 months. Similarly, on the basis of cross-sectional studies, it has been assumed that salivary gland viruses of rodents produce long term chronic infection of the salivary gland, but there has been little experimental basis for this assertion and no estimate of duration of infection. This report describes a year's study of subclinical mouse salivary gland virus (MSGV) infection. In view of marked biological and physical similarities between mouse and human salivary gland viruses(2), it was believed that a study of chronicity with MSGV would serve as experimental model for human salivary gland virus.

*Materials and methods.* Virus was obtained from Drs. Margaret G. Smith and Richard S. Lee, St. Louis, and maintained by passage of submaxillary salivary gland suspensions inoculated subcutaneously into the region of submaxillary glands of NIH "General Purpose" Swiss mice, a strain free of spontaneous salivary gland virus infection(2). Forty male "General Purpose" mice 3 weeks old were inoculated intraperitoneally with 0.2 ml of $10^{-3}$ dilution of infected mouse salivary gland suspension of the 30th mouse passage representing approximately $10^2$ to $10^3$ $TCID_{50}$ doses. Mice were maintained for a year during which time, at given intervals, 2 to 4 mice were removed for study. Mouth swabs, urine and submaxillary glands were obtained from individual mice. Trypsin dispersed mouse embryo tissue culture tubes were used for all virus isolations. Cultures were prepared from whole 14-16 day mouse embryos, grown in 10% human serum in Eagle's basal medium, and maintained in 5% horse serum in Eagle's medium; media contained penicillin and streptomycin. The mouth swab was rinsed in a vial containing 2 ml of Hanks' medium, and 0.5 ml transferred to each of two tissue culture tubes. Urine was diluted with one ml of Hanks' medium and 0.1 ml inoculated into each of 2 tubes. Ten percent suspensions of the salivary glands were prepared and 0.1 ml of 10-fold serial dilutions inoculated into each of 2 culture tubes. Cultures were observed for at least 2 weeks. Virus isolations were identified by the characteristic pattern of cytopathic effects and typical intranuclear inclusions(3). Virus titers are expressed as number of tissue culture doses/0.1 ml of 10% gland suspension. Glands were fixed in Zenker's-formalin and stained with hematoxylin-eosin. Slides were carefully examined for intranuclear inclusions by at least two observers.

*Results.* No evidence of overt disease was seen in any of the 40 mice. Results of virus recovery and pathologic studies are summarized in Table I. The first isolation of MSGV from the submaxillary gland, on eighth day after inoculation, coincided with appearance of virus in mouth swabs and of inclusions in the submaxillary gland. Maximum titer was obtained on 12th day; this was the only time at which virus was recovered from urine. After one year the submaxillary glands of 3 mice were still positive for MSGV and of the 2 mice tested both were excreting virus in the saliva.

Inclusion bodies were most numerous during early stages of infection. After 60th day, no inclusions could be demonstrated in submaxillary glands of any of the 9 mice studied although all had virus in the glands in titers of $10^2$ to $10^5$. There appeared to be no definite correlation between virus titer and number of inclusions. However, further studies would be needed to establish this point.

*Discussion.* Large doses (approximately $10^6$ $TCID_{50}$) of MSGV are required to produce fatal infection of adult mice after intraperitoneal or intracerebral inoculation, but subclinical infection is established regularly with as little as 1 to 10 $TCID_{50}$(2). The dose used in the experiment reported here produced no overt disease, but produced chronic subclinical infection of submaxillary salivary

TABLE I. Virus Isolation Attempts and Presence of Inclusion Bodies in Individual Mice Sacrificed at Intervals after Intraperitoneal MSGV Inoculation as Weanlings.

| Days after inoculation | Virus titer* in submaxillary gland, Mouse No. 1 | 2 | 3 | 4 | Inclusion bodies in submaxillary gland, Mouse No. 1 | 2 | 3 | 4 | Virus in mouth swab, Mouse No. 1 | 2 | Virus in urine, Mouse No. 1 | 2 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | $<10^0$ | $<10^0$ | | | 0 | 0 | | | 0 | 0 | 0 | 0 |
| 4 | ” | ” | | | 0 | 0 | | | 0 | 0 | 0 | 0 |
| 6 | ” | ” | | | 0 | 0 | | | 0 | 0 | 0 | 0 |
| 8 | $10^4$ | $10^4$ | | | + | + | | | + | + | 0 | 0 |
| 12 | $\geq 10^6$ | $\geq 10^6$ | | | ++ | ++ | | | + | + | + | + |
| 18 | $10^4$ | $10^4$ | | | +++ | +++ | | | + | + | 0 | 0 |
| 36 | $10^5$ | $10^3$ | | | +++ | 0 | | | + | + | 0 | 0 |
| 60 | $10^3$ | $<10^0$ | | | ++ | 0 | | | 0 | 0 | 0 | 0 |
| 120 | ” | $10^2$ | $10^5$ | $10^4$ | 0 | 0 | 0 | 0 | | | | |
| 240 | ” | $10^3$ | | | 0 | 0 | | | | | | |
| 365 | $10^2$ | ” | $10^2$ | | 0 | 0 | 0 | | + | + | 0 | 0 |

* Titer expressed as No. of tissue culture doses/0.1 ml of 10% gland suspension.

glands of at least one year's duration. On the basis of inclusion bodies, one might have concluded that the infection disappeared between the 60th and 120th days, but virus was present in moderate titer from 120th to 365th day, and at least some mice were still excreting virus in the saliva. This finding implies that absence of inclusion bodies in adult human salivary glands should not *per se* be considered proof of the absence of virus; however, attempts to recover virus from saliva and parotid glands of a small number of adult humans have been unsuccessful(1, and unpublished). Failure to recover virus from urine of mice after the acute phase of infection is in marked contrast to the high frequency of recovery of human salivary gland virus from urine of children months after initial infection. This may be related to failure of the mouse virus to produce lesions in the renal tubules(4), as opposed to their frequent presence in infected children(5).

Our data indicate that MSGV would provide a rather unique experimental model for study of a localized, subclinical virus infection. Of particular interest would be a study of experimental conditions that might activate the virus to produce disseminated disease. Such a study might have bearing upon human cytomegalic inclusion disease since dissemination of the human salivary gland virus is often noted in the presence of stress conditions such as prematurity, pertussis, and malignant disease(5).

*Summary.* Inoculation of weanling mice with a sublethal dose of MSGV produced chronic, subclinical infection of submaxillary gland of at least one year's duration. Virus was isolated from urine during the acute stage of infection and from saliva during both acute and chronic stages. Inclusion bodies in the salivary gland were not seen after the 60th day of infection.

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# Proteins of Heart in Experimental Cardiac Hypertrophy in the Rat.* (24452)

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Rapid cardiac hypertrophy follows exposure of rats to a reduced atmospheric pressure with increased environmental temperature(1). For example, after exposure to these conditions for 4 hours a day, a 50% increase in heart weight occurs after 12 days. A procedure has been developed for quantitative determination of cardiac protein fractions(2). The findings on growing rats have been reported(3). It was desirable to investigate the protein fractions of the hypertrophic hearts.

*Methods.* Hypertrophy was produced as previously described(1). Female rats of the Addis-Slonaker strain weighing 150 g were exposed to a simulated altitude of 25,000 ft. at 100°F for 4 hours a day. After average accumulated exposure time of approximately 48 hours, the mortality was 40%, deaths occurring mainly during the first 8 hours. The morning following last exposure, the animals were sacrificed and the hearts prepared as previously described(2), without the auricular appendages. Protein fractionation was carried out using procedure A. Briefly, the steps include the following: (1) grinding with sand; (2) extraction with 0.28 M Sucrose, .01 M Versene at pH 5 and centrifugation at 19,000 g (supernatant Fraction I); (3) extraction of residue with 0.28 M Sucrose, .01 M Versene at pH 7.4 and centrifugation at 650 g followed by 19,000 g (supernatant Fraction IA and residue mitochondria Fraction III); (4) extraction with 0.6 M KCl phosphate-pyrophosphate-versene buffer (supernatant Fraction II) and centrifugation to obtain residue (R). Fractions I, IA and II were subfractionated electrophoretically with Kern microelectrophoresis apparatus (type LK 30) which utilizes the interference principle. No unusual difficulties were encountered in analysis of the hypertrophic hearts. It was observed, however, that Fraction I, which is normally only slightly tinged with pink, was now distinctly red. Fraction II was considerably more turbid than usual but did not appear to be more viscous.

*Results.* In general, weight did not change during exposure to experimental conditions. Animals with weight loss exceeding 5 g were discarded. Fourteen hypertrophic hearts from 150 g rats with average weight of 794 mg were analyzed. The expected heart weight for unexposed animals was 541 mg. The findings were compared with the predicted values for 150 g rats obtained from the regression equations previously derived(3). The results are shown in Table I.

The total protein increased 42%. Individual protein fractions grew in a disproportionate manner. Several protein fractions exceeded general growth of the organ. Fractions $I_5$, II and mitochondria (III) exhibited the largest increases.

TABLE I. Quantitative Analysis of Protein Fractions from Hypertrophic Hearts.

| | Pobs. (mg) | P 150 (mg) | Pobs./P 150 | $R\%_{150}$ |
|---|---|---|---|---|
| $P_t$ | 114.7 ±3.1* | 80.7 | 1.42 | .52 |
| $I_t$ | 24.5 ± .9 | 16.9 | 1.45 | .62 |
| $I_1$ | 3.0 ± .1 | 2.8 | 1.07 | .53 |
| $I_2$ | 3.5 ± .2 | 2.4 | 1.46 | .65 |
| $I_3$ | 3.9 ± .2 | 2.9 | 1.34 | .72 |
| $I_4$ | 4.9 ± .3 | 3.4 | 1.44 | .51 |
| $I_5$ | 9.2 ± .4 | 4.6 | 2.00 | .67 |
| $IA_t$ | 9.0 ± .4 | 6.9 | 1.30 | .41 |
| $IA_1$ | 2.1 ± .1 | 1.8 | 1.17 | .43 |
| $IA_2$ | 4.5 ± .2 | 3.3 | 1.36 | .52 |
| $IA_3$ | 2.4 ± .2 | 1.7 | 1.41 | .27 |
| $II_t$ | 30.2 ±1.3 | 20.0 | 1.51 | .84 |
| $II\alpha$ | 5.2 ± .3 | 4.5 | 1.16 | .90 |
| $II\beta$ | 19.7 ± .8 | 11.0 | 1.79 | 1.00 |
| $II\gamma$ | 5.3 ± .5 | 3.9 | 1.36 | .63 |
| III | 9.6 ±1.1 | 5.6 | 1.71 | .62 |
| R | 41.0 ± .9 | 30.6 | 1.34 | .30 |

* Stand. error.

$P_t$ = total protein; $I_t$, $IA_t$, $II_t$, III and R refer to total protein of major fractions; other subscripts refer to specific subfractions. Pobs. = experimental values; P 150 = calculated from regression equations[3] for 150 g normal rats; Pobs./P 150 = ratio of value for hypertrophic heart to normal heart; $R\%_{150}$ = increase in protein (mg)/g gain in body wt calculated from equations[3].

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† Investigator, Howard Hughes Fn.

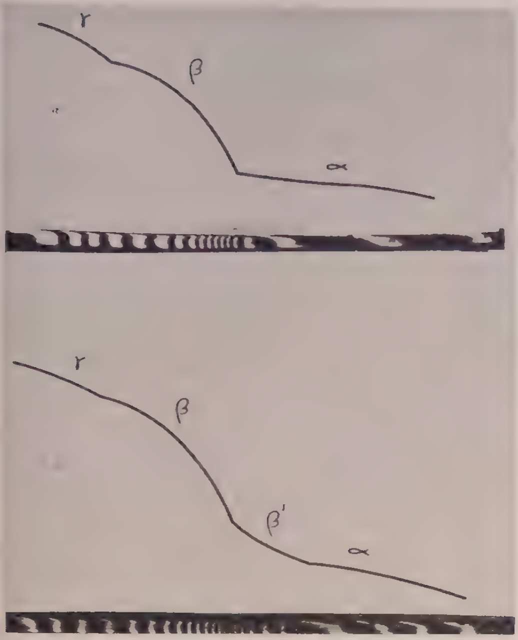

FIG. 1. Fraction II for Normal Heart (a) and Hypertrophic Heart (b). Extrapolation of electrophoretic pattern obtained with a Kern microelectrophoresis apparatus.

Close inspection of the interference patterns suggested that $I_5$ contained a second component. The possibility that this was due to myoglobin was offered by the following: It was pointed out above that Fraction I of the hypertrophic hearts was distinctly red. This suggested that an increase in hemoglobin and/or myoglobin had taken place. To test this point, the concentrated Fraction I was subjected to electrophoresis on paper at pH 8.9 with buffer using Spinco Electrophoretic apparatus. Unstained strips were compared to strips stained in the usual manner with brom thymol blue. Examination of these strips indicated that the pigment moved with the slowest moving Fraction $I_5$. The pigment had 2 components. By comparison with rat hemoglobin the faster pigment component was ascribed to this substance, the slower was assumed to be myoglobin. If this is the case it must be concluded that some increase in hemoglobin and a considerable increase in myoglobin content had taken place. Nearly undetectable amounts of pigment were present in normal hearts under similar conditions.

Fraction II $\beta$ exhibited a second component with hypertrophy (Fig. 1). The fractions of II designated $\alpha$, $\beta$ and $\gamma$ in this laboratory follows Dubuisson's designation $\alpha$, $\beta$ and contractin. II $\beta$ corresponds to actomyosin of other investigators.

*Discussion.* The data describe changes in protein fractions of the heart in which hypertrophy was induced due to an increase in work load. It was not expected that the information would be useful in interpreting the events which had taken place since specific functions have not yet been assigned to each fraction. Nevertheless the apparent increase in myoglobin and the factual increase in mitochondria indicate an expansion of the oxygen transporting and oxygen utilizing systems. The changes in II $\beta$ indicate presence of increased quantities of contractile protein. These findings are consistent with suggestions made previously that during metabolic disequilibrium changes in protein content are governed by the functional demand for services of the individual proteins which take place during the change(3).

It was of interest to compare changes observed with hypertrophy to that seen in growth. From data previously reported(3) the net deposition of protein for each fraction/g gain in body weight for 150 g rats was calculated (column 5, Table I). Comparison of this data with that derived from hypertrophic hearts suggested that hypertrophy is not simply an exaggerated growth. Certain differences were quite striking. For example Fraction II $\alpha$ was increased rapidly during normal growth but not with hypertrophy. Evidently the increased demand for the services of each fraction during an induced hypertrophy under an increased work load differs in certain respects from the demands made during normal growth.

*Summary.* Protein fractions of hearts from rats in which cardiac hypertrophy was produced by exposure to elevated environmental temperature and reduced atmospheric pressure were studied. Increases occurred in all fractions but in a disproportionate manner. Largest increases were observed in the following fractions: (1) $I_5$, due in part to an apparent increase in myoglobin, (2) in the contractile Fraction II $\beta$, due in part to appear-

ance of a component not normally demonstrated, and (3) in the mitochondrial fraction. It is suggested that these changes represent an adaptation to work demands placed upon the heart under experimental conditions.

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## Brain Excitability in Relation to Age and Hypophysectomy. (24453)

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A type of brain excitability may be determined by measuring minimal electroshock threshold (EST) of rats. A number of variables have been reported to change this response: body weight, nutritional factors, endocrine imbalances, etc.(1-5). The undefined significance of body weight and of other variables occurring in chronic experiments makes it difficult to obtain, by EST measurement, an exact perspective of the role of the endocrine system on sensitivity of the central nervous system, and this may account for disparities in conclusions(2-5). In an attempt to explain the interrelationship of EST with body, brain, and adrenal weights, and age in intact growing rats, and in early and late hypophysectomized rats the following experiments were performed.

*Materials and methods.* Two hundred and eight, male Sprague-Dawley rats were used. Body weight and EST measurements were made on each of 10 groups of 10 intact rats, on each of 8 groups of 10 early hypophysectomized rats, and on each of 2 groups of 9 and 2 groups of 10 late hypophysectomized rats. Intact animals were exposed to electrical stimulation only once at 10 different time intervals between 23 and 95 days of age; the early hypophysectomized rats at 8 intervals between 25 and 137 days of age; and the late hypophysectomized rats at 4 intervals between age 70 and 137 days. Electroshock was produced by a 60-cycle stimulator with a 0.16 second duration through corneal electrodes. Tissues were removed and weighed 30 minutes after electroshocking. Coefficients of correlation and regression between EST and the other factors examined (organ and body weights and age) were statistically evaluated.

The results are summarized in Table I. Intact animals showed an increase in body, brain, liver, and adrenal weights; also EST increased with age. Early hypophysectomized rats (operated at 23 days) exhibited an initial drop in body weight followed by an increase. Brain and adrenal weights decreased; whereas liver weights and EST increased, although less rapidly than in intact animals. Late hypophysectomy (operation at 67 days) suppressed growth; body and brain weights remained steady, but adrenal and liver weights decreased. The adrenals appeared to atrophy whereas EST continued to increase parallel to rate of increase observed in the intact animal.

*Discussion.* In intact, growing animals, absolute value of EST increases with age and consequently with body, and other organ weights. In the hypophysectomized animals, *in which body and brain weight increase is inhibited,* EST still increases with time. When EST values were plotted against age, straight lines resulted; their coefficients of regression have been calculated by standard statistical methods. At 95% confidence intervals, there was no significant difference between the coefficients for intact (0.362) and late hypophysectomized rats (0.324). On the other hand, this coefficient was significantly different (0.220) for early hypophysectomized animals. Age appears to be most directly related to EST in both hypophysectomized and normal animals. Instead, if EST

TABLE I. Body, Brain, Liver, and Adrenal Weight and EST Means in Rats after Exposure to Single Shock.

| Age (days) | No. of rats | Body wt, g ± S.D. | | Brain wt, mg ± S.D. | | Liver, mg ± S.D. | | Adrenals (2), mg ± S.D. | | EST, ma ± S.D. | | EST, ma/100 g body wt |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Intact | | | | | | | | | | | | |
| 23 | 10 | 51.3 | 3.3 | 1320 | 33 | 2413 | 375 | 21.5 | 1.4 | | | |
| 25 | ” | 50.2 | 2.5 | 1347 | 43 | 2275 | 148 | 15.7 | 1.6 | | | |
| 28 | ” | 65.9 | 3.2 | 1383* | | | | | | 16.7 | .1 | 25.2 |
| 39 | ” | 128.4 | 6.7 | 1524 | 96 | 6895 | 644 | 26.6 | 2.5 | | | |
| 53 | ” | 210.3 | 10.9 | 1557 | 170 | 12859 | 540 | 32.1 | 5.8 | 20.8 | .5 | 9.75 |
| 67 | ” | 264.1 | 15.0 | 1660 | 57 | 13486 | 1004 | 38.6 | 3.9 | 21.1 | .6 | 8.55 |
| 70 | ” | 272.6 | 17.9 | 1577 | 270 | 13823 | 1001 | 40.1 | 4.2 | 24.1 | .8 | 8.9 |
| 81 | ” | 284.4 | 10.2 | 1568 | 15 | 13765 | 1500 | 43.5 | 5.6 | 29.7 | .5 | 10.5 |
| 95 | ” | 315.3 | 20.0 | 1582 | 42 | 14497 | 1625 | 49.0 | 6.2 | | | |
| Early hypophysectomized (23 days) | | | | | | | | | | | | |
| 25 | 10 | 48.5 | 4.1 | 1424 | 97 | 2302 | 233 | 14.5 | .4 | | | |
| 28 | ” | 57.3 | 5.6 | 1436* | | | | | | 16.3 | .2 | 28.2 |
| 39 | ” | 69.0 | 7.4 | 1484 | 54 | 3228 | 386 | 8.1 | 1.7 | | | |
| 53 | ” | 68.9 | 5.2 | 1476 | 124 | 3504 | 407 | 8.6 | 2.8 | 18.6 | .5 | 27. |
| 70 | ” | 73.3 | 4.1 | 1450 | 77 | 3371 | 397 | 8.3 | 2.0 | 19.2 | .7 | 26. |
| 81 | ” | 70.1 | 2.9 | 1481 | 77 | 3708 | 255 | 9.6 | 1.5 | 21.3 | .5 | 30. |
| 95 | ” | 76.2 | 8.6 | 1478 | 84 | 3484 | 461 | 7.9 | .9 | 21.4 | .9 | 28. |
| 137 | ” | 91.1 | 7.5 | 1463 | 63 | 4263 | 733 | 9.5 | 2.3 | 27.8 | 1.1 | 30. |
| Late hypophysectomized (67 days) | | | | | | | | | | | | |
| 70 | 9 | 220.2 | 17.0 | 1545 | 89 | 10333 | 793 | 31.9 | 4.4 | 21.4 | .9 | 9.7 |
| 81 | 10 | 207.2 | 19.1 | 1581 | 78 | 8401 | 618 | 18.1 | 4.0 | 26.0 | 1.4 | 12.5 |
| 95 | ” | 209.3 | 18.3 | 1579 | 63 | 9089 | 1178 | 14.2 | 1.8 | 32.6 | 1.9 | 15.6 |
| 137 | 9 | 215.0 | 14.7 | 1539 | 157 | 9085 | 1420 | 15.1 | 1.0 | 42.3 | 2.7 | 19.7 |

* Interpolated values.

is represented in terms of body weight, the values obtained would be distributed in disorder, as would be expected if one assumed that the electric current applied through the corneal electrodes acted in a closed system, involving the CNS and not the total body mass. In normal animals, there is a possibility that the mass of the brain may be one of the factors responsible for EST increase. However, the EST-brain weight relationship is certainly not a direct one (Table I). It cannot be excluded that EST may be influenced by brain weight as well as age. The first factor could act through a mass effect on general uptake of the current; the second (age) through a more specific cellular variation, which would be influenced by the endocrine system. If brain weight influenced EST, then the values obtained in hypophysectomized rats (in which this weight did not significantly change) represented a higher relative threshold in comparison to the controls. The EST-Age correlation coefficient following early hypophysectomy leads to the conclusion that, when the animal is more affected by the hormonal deficiency, as in young, growing animals, the threshold is more susceptible to reduction. This may be related to some fundamental process involving neurons, parallel to the general lack of development.

The present findings, showing gradual increase of EST in the nonoperated animal as time increases, would be sufficient to account for the difference of threshold before and after hypophysectomy; this observation in the past(*2,3*) has been interpreted as a hypoexcitability induced by the deficiency status. In a general way, inability to change one set of variables (*e.g.*, hormonal influences of hypophysectomy) and maintain the others (body and organ weights or age) in the same condition as the controls, makes interpretation of the findings obtained on the convulsive threshold under the influence of endocrine deficiencies more difficult than has been assumed.

*Summary.* An investigation was carried out in Sprague-Dawley, male rats to determine relationship of total body, brain, adrenal, and liver weights, and age on EST in intact growing, and in early and late hypophysectomized rats. 1. EST is progressively

increased in relation to age in intact growing, and in early and late hypophysectomized rats. 2. Statistical analysis of values obtained shows that regression coefficients of Age vs. EST of intact growing and of late hypophysectomized rats are almost identical, whereas those of early hypophysectomized rats are lower. It is possible that early hypophysectomized rats become more sensitive to the stimulus than intact or late hypophysectomized animals. 3. The conclusion is reached that other variables, particularly age, have to be considered in seeking information of the effect of deficiency state itself on EST, or more generally on central nervous system sensitivity. On this basis, many previous conclusions require revision.

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## Studies in Histochemistry LIII. Microbiological Assay in Quantitative Histo- and Cytochemistry.* (24454)

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Quantitative chemical analysis of histologically or cytologically defined samples often requires micro technics to deal with the small quantities involved. Microbiological assay, the method of choice for certain B vitamins etc., has a remarkably great sensitivity which is well suited to the exacting requirements of histo- and cytochemical studies. Nevertheless, this analytical approach has been surprisingly neglected, and in fact only an occasional micro adaptation for any purpose has been made, *e.g.,*(1-4). Bioautography offers a sensitive means of detection but without the degree of quantitation afforded by the usual microbiological assay. Excellent comprehensive reviews of these technics have just appeared(5,6). Recent instrumental developments have made feasible the extension of microbiological assay to a scale requiring 500 to 1000 times less sample without loss in accuracy or precision. In this communication such a technic will be described with application to measurement of biotin, liberated from bound form by acid hydrolysis, and pantothenate, liberated by enzyme hydrolysis.

*Materials and methods.* The micro equipment employed has been described(7). Pyrex culture tubes 28 mm long, 4 mm inner diameter, with sleeve-type rubber serum-bottle stoppers were used. Constriction pipettes were employed for all volumes under 100 $\mu$l and mixing in the tubes was carried out by "buzzing." A constant temperature water bath with a plastic plate containing holes, 6 mm diameter, to hold the culture tubes served as the micro incubator. The plate was suspended 15 mm under the water surface so that the tubes were submerged but the tops of the stoppers were not. An ordinary well-controlled incubator can be used instead. A micro centrifuge (Microchemical Specialties Co., Berkeley) was also used. Growth was determined turbidimetrically at 650 m$\mu$ with a Beckman DU spectrophotometer. Lowry-Bessey micro cuvettes were employed for larger volumes (100 $\mu$l) and Glick-Grunbaum inserts were used with these cuvettes for small volumes (6 $\mu$l). *Lactobacillus arabinosus,* strain 17-5, with "double strength"

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* This study was supported by research grants from Eli Lilly and Co., Indianapolis, U.S.P.H.S., and Louis W. and Maud Hill Family Fn.

Skeggs-Wright medium was used for assay of biotin(8) and pantothenate(9). For both vitamins substitution of cysteine for cystine and addition of 1 mg folic acid per liter of medium was found to give more consistent results. For inoculum cultures, 0.025 μg % biotin or 2 μg % calcium d-pantothenate was added to the medium diluted with an equal volume of water. Stock cultures were maintained on APT Agar, No. 229 (Case Laboratories, Chicago). When the sample was a microtome section of fresh-frozen tissue, it was frozen-dried at –20°C in a vacuum desiccator over silica gel(7), weighed on a quartz fiber micro balance, and then subjected to hydrolysis. For biotin, optimal conditions for hydrolysis were found to be autoclaving the dried sample, *e.g.*, about 20 μg from a fresh section of rat liver 2 mm diameter and 20 μ thick, in 5 μl of 2 N sulfuric acid in a stoppered tube for 1 hour at 120°C. The hydrolysate was neutralized with 5 μl of 2 N sodium hydroxide and diluted with sufficient (usually 180 μl) 0.015 M phosphate buffer, pH 7, to bring the biotin concentration into the region required by a standard calibration curve. For pantothenate in the dried sample, *e.g.* liver as above, enzyme hydrolysis, adapted from the procedure of Novelli and Schmetz(10), required 18 hours at 37°C in a mixture consisting of 5 μl (2 Schmidt-Thannhauser units) of 0.2% purified intestinal alkaline phosphatase (Pentex, Kankakee, Ill.), 10 μl of 10% suspension of Dowex-treated pigeon liver acetone powder (Pentex) in 0.02 M potassium bicarbonate, 5 μl of 0.1 M Tris buffer, pH 8.4, and 30 μl water. The enzymes were inactivated by immersing the tubes in boiling water for 45 seconds and the hydrolysate then diluted with 100 μl of water. Before assay the hydrolysates of both vitamins were clarified by centrifugation for several minutes at 7500 g or greater. Sterile standard solutions of biotin in 0.015 M phosphate buffer, pH 7, were used in the range 0.1-1.0 mμg per ml. The calcium d-pantothenate standards were in the range of 10-100 mμg/ml water. Aliquots of 10 μl of standard or hydrolysate solution were employed for each assay, and these were set up in triplicate for each dilution. Hydrolysates were diluted 2 or 3 times and all dilutions were assayed simultaneously. Ten μl of inoculated medium were added to each tube and the mixture incubated at 30°C for 24 hours (pantothenate) or 48 hours (biotin). Finally 100 μl of 0.9% sodium chloride were added, the solution mixed and the absorbance measured.

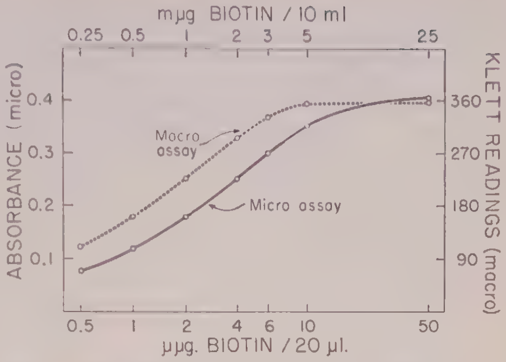

FIG. 1. Response of *Lactobacillus arabinosus* (17-5) to biotin in macro (10 ml/tube) and micro (20 μl/tube) assays. Bacterial suspension was not diluted before measurement in the former, but it was (1:1) in the latter.

*Results.* The data presented in Fig. 1 illustrate the close correlation between the macro and micro assays for biotin. The same degree of correlation obtains for pantothenate assays. To test the precision of the methods 10 aliquots from a standard solution of biotin and 10 from one of coenzyme A were analyzed and the respective means 4.05 μμg (% standard deviation and error, 3.2 and 1.0) and 22.6 mμg (% standard deviation and error, 4.9 and 1.5) were obtained. Triplicate analyses of hydrolysates of individual microtome sections of rat liver, dry weight 21-24 μg, gave a mean of 3.14 μμg biotin per μg dry weight for 10 sections (% standard deviation and error, 4.4 and 1.6) and 0.28 mμg pantothenic acid per μg dry weight for 7 sections (% standard deviation and error, 7.9 and 2.8).

Preliminary work indicates that the method for biotin can be used for niacin in the range 0.006-1 mμg by replacing the niacin in the double-strength medium by 0.1 μg % biotin. Hydrolysis requires autoclaving for 15 minutes at 120°C in 0.1 N sulfuric acid; 0.1 N sodium hydroxide is used for neutralization.

*Summary.* Extension of microbiological assay methods to a scale requiring 1/500th to 1/1000th the sample ordinarily used is described with specific application to the measurement of biotin and pantothenate in tissue sections.

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## Antimycobacterial Activity of Extracts of Rat Peritoneal Cells.* (24455)

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Lurie concluded (a) that acquired immunity to tuberculosis is merely enhanced natural resistance, and modes of action of the 2 phenomena are probably the same(1,2) and (b) that altered physiological reactivity of mononuclear phagocytes of the immunized animal is the most important single factor in resistance to the disease(3). These conclusions have gained considerable support. It appeared possible therefore that comparative studies of some physiological activities of mononuclear cells from animals of differing susceptibility to this disease might contribute to a better understanding of mechanisms of restriction of intracellular mycobacterial growth. As a part of such studies, antimycobacterial activities of extracts of cells of induced peritoneal exudates from the highly susceptible guinea pig and the very resistant rat have been investigated. It seems desirable to report preliminary results of these experiments.

*Materials and methods.* Male albino guinea pigs of the "English" breed weighing 800 to 1200 g each, and 2 strains of male albino rats of 250 to 400 g weight were used in groups of 8 to 18 animals for each experiment. Exudate induced by intraperitoneal injection of glycogen(4) was collected on sixth day, at time of monocyte-type cell predominance, by washing peritoneal cavities with large quantities of isotonic 0.005 M Tris buffer, pH 7.4. Fluid was collected by gravity from living guinea pigs, and from rats by exposing peritoneal cavities immediately following their death from sharp head blows. Siliconed glassware or lusteroid or polyethylene containers were used for handling all cell suspensions. About 1 to 2.5 l of pooled cell suspension was obtained from each group of animals. The number of cells was determined by hemocytometer for preliminary standardization. Cells were concentrated in 20 to 40 ml of isotonic Tris buffer in a refrigerated centrifuge. Concentrated cell suspensions were freed from contaminating erythrocytes, if any, by recentrifuging and suspending 10 minutes in hypotonic NaCl solution, which was then made isotonic by addition of an equal volume of hypertonic NaCl solution(5). Total and differential cell counts were made and the suspension recentrifuged; thus cells were washed twice after removal from animals. The button of cells was evenly suspended in glass-distilled water to make concentrations of about 60 to 70 x $10^6$ cells/ml and suspensions were subjected to sonic vibration in Raytheon

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TABLE I. Antimycobacterial Activity of Mononuclear Cell Extracts. Figures refer to average number of colonies in 4 weeks from replicate tests of 0.02 ml of filtrate-bacteria mixtures. Test organism: *Mycobacterium tuberculosis*, var. *hominis*, Strain H37Ra.

| | | Hours of contact with bacteria: | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 24 | 48 | 0 | 1 | 24 | 48 |
| *Exp. 1:* | | Rat cell extract (Wistar) | | | | $H_2O$ | | | |
| Filtr.-bact. mix.: | Undil. | >250 | >250 | 250 approx. | 0 (6 repl.)*<br>1 (1 plate)*<br>2 (1 " )* | >250 | >250 | >250 | 1+ ( 8 repl.) |
| | Dil. 1:5 | 67 | 60 | 52 (5 repl.) | 0 (9 repl.)* | 59 | 55 | 68 | 98 (14 " ) |
| | | No. mammalian cells, 3000<br>No. bacterial cells, 1 | | | | | | | |
| *Exp. 2:* | | Rat cell extract (CFN) | | | | $H_2O$ | | | |
| Filtr.-bact. mix.: | Undil. | >300 | >300 | 20 (5 repl.)* | 0 (4 repl.)* | >300 | >300 | >300 | 1+ |
| | Dil. 1:5 | 122 | 107 | 5 (7 " )* | 0 (6 " )* | 114 | 111 | 103 | 155 ( 9 repl.) |
| | | No. mammalian cells, 1200<br>No. bacterial cells, 1 | | | | | | | |
| *Exp. 3:* | | Guinea pig cell extract | | | | $H_2O$ | | | |
| Filtr.-bact. mix.: | Undil. | >150 | >150 | >150 | >150 | >150 | >150 | >150 | >150 |
| | Dil. 1:5 | 32 | 34 | 36 | 34 (9 repl.) | 32 | 33 | 30 | 44 (16 repl.) |
| | | No. mammalian cells, 5000<br>No. bacterial cells, 1 | | | | | | | |

* No increase in No. of colonies after 6 weeks' incubation.

9 Kc sonic oscillator until microscopic examination revealed only granules with no whole cells (5 to 10 minutes). Glass-distilled water was then added to make suspensions correspond to 45 to 55 x $10^6$ cells/ml, sediment was removed by 1 hour's centrifuging at 20,000 g and 1.5 to 2.5 ml of supernate was sterilized by filtration through a millipore (MF) pad using the Swinney adapter. A corresponding quantity of glass-distilled water was similarly filtered as control for each experiment. Antibacterial tests were performed as follows: Test organisms: *Mycobacterium tuberculosis* var. *hominis*, strain H37Ra. Five-day cultures in Proskauer and Beck type medium containing bovine serum albumin and Tween 80® for dispersed growth, were centrifuged lightly to remove clumped bacteria. The top layer of supernatant was diluted 1:200 in 0.005M phosphate buffer, pH 6.8, containing albumin and Tween 80®. Diluted culture was added to cell extract or water filtrate in 1 to 4 parts. Immediately, 0.02 ml amounts of each mixture were cultured in replicate on coagulated egg medium. Filtrate-bacteria mixtures were then diluted 1:5 in phosphate buffer-Tween 80®-albumin and similarly cultured in replicate. When surface of medium was dry, plates were sealed in polyethylene envelopes or saran wrap and incubated for growth. Replicate cultures of filtrate-bacteria mixtures were repeated after 1, 24 and 48 hours at 37°C. Colonies were counted at intervals after 2 to 6 weeks' incubation.

*Results.* It is evident from Table I that many tubercle bacilli were rendered non-cultivable after 24 hours' contact with extract of normal rat cells and that 48 hours' contact resulted in practically complete inhibition. In contrast, 48 hours' incubation in normal guinea pig cell or distilled water filtrate had no effect; the number of bacteria in water controls appeared to increase slightly.

Extract of a relatively large number of mammalian cells was employed per single bacterial cell. Rat cell filtrate effectively inhibiting bacteria in a ratio of 1200 to 1, lost its inhibitory effect under our conditions when diluted 10 times prior to addition of bacteria.

Differential counts (Giemsa stained, at least 500 cells counted) revealed that 90 to 95% of cells from both animal species were "large mononuclear" cells. Suspensions from rats, however, were characterized by 4 to 6% large granular basophilic mast cells, absent in guinea pig cell preparations. The latter more

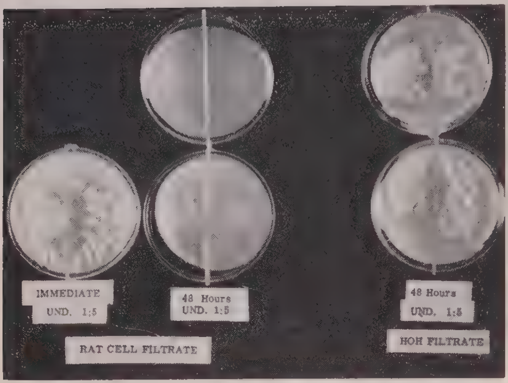

FIG. 1.

characteristically contained a comparatively larger percentage of eosinophilic-staining macrophages than those from rats.

Analyses for protein content by biuret and microkjeldahl methods indicated that soluble cell material (*i.e.*, supernate of centrifuged lysates) from rat cell preparations had somewhat less protein (corresponding in different groups of rats to approximately 40 mg/1 x $10^9$ cells of original suspensions) than guinea pig cell preparations (about 50 mg/1 x $10^9$ cells). Membrane filtration for sterilization resulted in only a slight decrease in protein content of extracts of cells from both species.

*Discussion.* Most previous attempts to obtain antibacterial substances from leukocytes have been made with polymorphonuclear cells (see Skarnes and Watson(6) for review). However in a report concerned mainly with extracts of normal rat spleen, Bloom, Hudgins and Cummings(7) briefly described tuberculostatic activity of cells of peritoneal exudates obtained from rats 48 hours after injection of mineral oil. These exudates contained "large numbers of monocytes." The decrease in number of viable mycobacteria reported by these investigators appeared to be of the same order as that obtained in our experiments.

Rich stated that it had never been demonstrated that extracts of mononuclear phagocytes had a deleterious effect on survival or multiplication of tubercle bacilli(8). Although with certain bacteria it is not difficult to demonstrate a weak *in vitro* bactericidal effect of phagocytes by using concentrated extracts from masses of phagocytes, he believes that such activity is of little significance in killing of ingested bacteria *in vivo*. This view may be essentially correct since it appears likely that a combination of effects, rather than a single agent, is responsible for inhibition of intracellular bacteria by macrophages. One factor in a complex interreaction might be a growth-inhibiting chemical. Hirsch, for example, stated that at least 3 potentially bactericidal substances appear to be present in polymorphonuclear cytoplasm: acid, lysozyme and phagocytin, present information suggesting that these agents may act synergistically(9). The possibility of this renders the disproportion between numbers of mammalian and bacterial cells of less significance. Further work may indicate that amount of extract used in the present experiments is sufficient for a greater number of bacteria. Optimum conditions for maximum activity of this property of rat cell extracts have not yet been established. Hirsch has shown that number of bacteria present does not strikingly affect activity of phagocytin on enteric bacteria.

The main cytological difference between rat and guinea pig peritoneal exudates, *i.e.*, mast cells in the former, induces speculation concerning the source of the antibacterial property of these preparations. Should it prove to be the mast cell, the significance of this activity in destruction of intracellular bacilli by macrophages might be questioned. However it has been suggested that mast cells are merely polysaccharide-laden macrophages (Higginbotham and Dougherty, quoted by Padawer(10)). In this connection, it is interesting that many antibacterial substances extracted from tissues are of the nature of basic proteins or basic peptides(6).

*Summary and conclusions.* 1) Membrane-filtered water-soluble constituents of sonic-disrupted cells from peritoneal exudates of 2 strains of albino rats exert a definite inhibitory effect on cultivability of *Mycobacterium tuberculosis*, strain H37Ra, in phosphate buffer at pH 6.8 on 24 hours' contact at 37°C, inhibition being almost complete after 48 hours incubation. 2) Similarly treated extracts of cells from peritoneal exudates of normal guinea pigs standardized in the same manner have no effect. 3) Differential cell counts of preparations from which extracts were made indicated that 90 to 95% of cells in exudates from both species were "large mononuclear" macrophages. Rat exudates, however, contained 4 to 6% mast cells, absent in guinea pig exudates. The significance of mast cells in antibacterial activity noted was not determined in the work reported here.

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# Hyaluronate-Inactive Spreading Factor of Murine Endometrial Secretions (Uterone).* (24456)

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The spread of indicators such as india ink or hemoglobin in the dermis of test animals is increased by 2 types of so-called spreading factors(1). The first type is hyaluronate-active and includes hyaluronidase and other substances which act by depolymerization of hyaluronic acid gel; the second type is the hyaluronate-inactive spreading factors which do not exhibit this activity *in vitro* or in dead skin. Several mechanisms have been proposed to account for the spreading effect of hyaluronate-inactive spreading factors. These are: a) liberation of bound hyaluronidase from skin(2); b) action through alteration of another component of the dermal barrier, different from hyaluronic acid(3); or c) production of inflammatory exudate with subsequent edema(4).

This paper deals with observations made during studies on biologic activities of endometrial secretions (uterone) obtained by cervical ligation in mice(5), revealing that crude uterone enhances intradermal spread of india ink. Experiments were designed to quantitate the spreading effect of uterone and to elucidate its mechanism.

*Methods. Animals.* Mature male and female mice of RFM and BALB/c strains, 2-3 months of age, were kept in animal rooms of Jackson Memorial Laboratory and fed tap water and purina chow *ad libitum*. *Materials.* Uterone was obtained from mice of the same strain as that of test animals. Before its use in experiments with BALB/c mice, uterone was lyophilized and reconstituted with sterile Ringer's solution to concentrations varying from one-third to 9 times that of the original uterone. On completion of each experiment, sterility of uterone was ascertained by culture on blood agar plates to establish absence of extraneous bacterial spreading factors. India ink was diluted with Ringer's solution 1:3 and 1:7. Hyaluronidase was commercial preparation No. 214004 (Wydase, Wyeth), diluted in Ringer's solution to the desired concentration. Control serum, known to contain no spreading factor(3), was obtained from RFM mice and used fresh. *Procedures.* Spreading of india ink was determined by measuring area of spread of intradermally injected india ink, using a tuberculin syringe and gauge 27 needle to introduce the substance to be tested

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TABLE I. Comparison in RFM Males of Surface Areas of Ink Spots Resulting from Intradermal Spread of India Ink Suspended in Mouse Serum (RFM), Uterone (RFM) and Various Concentrations of Wydase.

| | | | Wydase | | | |
|---|---|---|---|---|---|---|
| | Serum | RFM uterone | 6 units | 12 units | 50 units | 100 units |
| Mean spread, cm² | 1.14<br>(1.02–1.40) | 1.87<br>(1.49–2.12) | 1.55<br>(.92–2.16) | 2.86<br>(2.60–3.08) | 3.40<br>(3.24–3.63) | 3.90<br>(3.30–4.62) |

( ) = Range.

together with india ink into a shaved area on flank of the mouse. Total injected volume was 0.1 ml. The animals were killed and skinned 12 hours after injection. The skin containing the ink bleb was placed on a transilluminated glass plate, subcutaneous tissue facing the observer, and its borders were traced on a sheet of transparent "Lucolite 11-6." The traced plastic areas were cut out and weighed. Conversion of weights thus obtained into area of spread was made by dividing weights of traced plastic areas by weight of plastic area measuring exactly 1 cm$^2$. 1. *Spreading effect of commercial hyaluronidase* was determined in 27 RFM males divided into 6 groups receiving india ink marker containing 6U, 12U, 50U, and 100U of hyaluronidase. The spread obtained was compared with that resulting from serum or Ringer's solution. 2. *Spreading effect of RFM uterone* was measured in 5 RFM males receiving india ink and fresh RFM uterone and was compared with results obtained in 6 RFM mice receiving india ink and serum. 3. *Spreading effect of BALB/c uterone* (Lyophilized) was measured in 34 BALB/c mice (19 males, 15 females). These animals were divided into 4 groups (females) and 5 groups (males) and given india ink marker and uterone made of the following concentrations: original concentration (2.43 g of solid/100 ml), and approximately one-third, one-half, 2 and 9 times the original concentration. Results were compared with the spread obtained in 3 males and 3 females with india ink and serum. 4. *Spreading effect of BALB/c uterone and commercial hyaluronidase combined.* Eight BALB/c males were given india ink and 6 units of hyaluronidase dissolved in lyophilized BALB/c uterone reconstituted to its original concentration. 5. *Spreading effect of BALB/c uterone in dead BALB/c skin.* India ink marker and BALB/c uterone reconstituted to 5 times its original concentration was injected into BALB/c skin one hour after killing the animal, and compared with spreading of india ink containing Ringer's solution and 15U of commercial hyaluronidase. The skin was fixed in formaldehyde 30 minutes later. 6. *Spreading of india ink in rabbit skin in relation to time as influenced by hyaluronidase, mouse uterone and ovomucoid.* Rabbits were used because they are the standard assay animal for spreading factor, and permit numerous spots to be studied and biopsied in the same animal in rapid succession. Flanks of male rabbits were shaved and india ink containing the following materials was injected: a. 30 units of commercial hyaluronidase; b. 15 units of commercial hyaluronidase; c. BALB/c mouse uterone 1.3 times original concentration or d. BALB/c mouse uterone, 9 times original concentration; e. ovomucoid, 2.5 g/100 ml; f. saline. The resulting spots were traced on plastic sheets placed over the skin at intervals of 1′, 2′, 5′, 10′, 20′, 60′, 90′, and 18 hours. Another rabbit was treated in a similar fashion, except that no india ink was used. In this animal 3 injections were made for each substance and biopsies were taken 5 minutes, 20 minutes, and 24 hours after injection. These tissues were fixed in formaldehyde and sections were prepared and stained with hematoxylin-eosin. A similar experiment was done in Swiss mice using uterone obtained from the same strain. 7. *In vitro assay of uterone for depolymerization of hyaluronic acid.* The viscosity reduction method described by J. Madinaveitia and T. H. H. Quibell(6) was followed using vitreous body hyaluronic acid as a substrate and an Oswald viscometer Kimble, size No. 200.

*Results* are shown in Tables I and II and Figs. 1 and 2. The results indicate that commercial Wydase enhances the spreading of india ink in the dermis of mice much as it

TABLE II. Comparison in BALB/c Males and Females of Surface Areas of Ink Spots Resulting from Intradermal Spread of India Ink Suspended in Mouse Serum (RFM), Uterone (Lyophilized BALB/c Reconstituted to Varying Concentrations) and the Same Uterone Reconstituted to Original Concentration Combined with 6 Units Wydase.

| | | BALB/c uterone | | | | | BALB/c uterone (1.1 N) + 6 u Wydase |
|---|---|---|---|---|---|---|---|
| | Serum | .3 N | .6 N | 1.1 N | 2.2 N | 9.0 N | |
| Mean spread in males, cm² | 1.24<br>(1.16–1.31) | 1.00<br>(.91–1.10) | 1.16<br>(1.08–1.20) | 1.57<br>(1.41–1.73) | 1.63<br>(1.27–2.10) | 2.47<br>(2.30–2.73) | 2.68<br>(2.03–3.40) |
| Mean spread in females, cm² | .95<br>(.75–1.09) | 1.40<br>(1.30–1.46) | 1.15<br>(.91–1.29) | 1.70<br>(1.63–1.74) | 1.69<br>(1.31–1.91) | | |

( ) = Range.

does in the rabbit which has previously been used as a test animal to measure spreading factors. The intradermal injection of murine uterone from BALB/c and RFM mice enhances spreading of india ink in BALB/c and RFM mice (Fig. 1 and Table I and II). BALB/c uterone potentiates spreading effect of Wydase in BALB/c male mice. The same BALB/c uterone which had these spreading effects *in vivo* did not enhance spreading when injected into dead BALB/c skin. Viscosimetric studies showed that BALB/c uterone does not depolymerize hyaluronic acid *in vitro*.

In rabbits, Wydase caused a rapid initial enhancement of spreading of india ink, whereas ovomucoid and uterone resulted in a much slower progressive enhancement of spread (Fig. 2). Histologic study in rabbits and mice revealed that this was accompanied by edema and inflammation of dermis while no such changes were seen with Wydase.

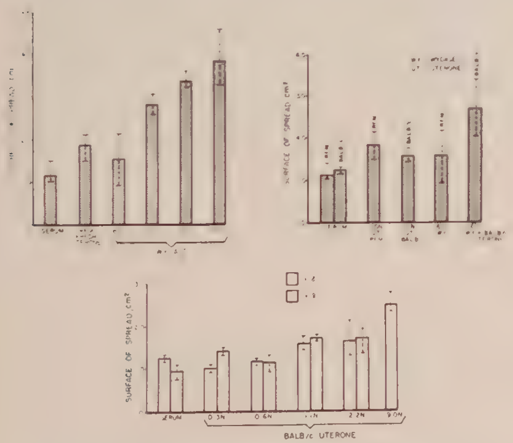

FIG. 1. Bar graph expressing avg area of spread of india ink (and range) following intradermal inj. of substances listed on abscissa.

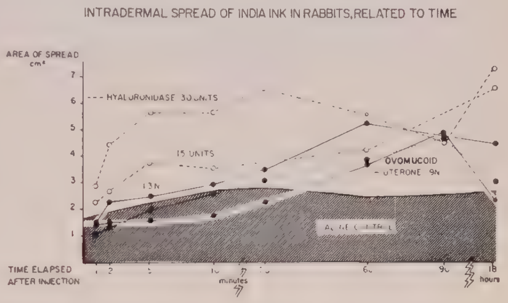

FIG. 2. Comparison of development of spreading area related to time following inj. of saline, hyaluronidase, ovomucoid and uterone with india ink marker into dermis of rabbit. Note rapid spreading of hyaluronidase during first minutes compared to slow and progressive spread caused by ovomucoid and uterone. Also note shrinkage of spreading area at end of experiment in case of uterone and ovomucoid.

*Discussion.* Murine uterone was shown to enhance spread of intradermal india ink and to potentiate the spreading effect of commercial hyaluronidase. This appears to be caused not by hyaluronidase activity, but by some other mechanism. Histologic observations made in rabbits and mice indicate that Hechter's explanation of edema and inflammation as hyaluronate-inactive spreading mechanisms applies to this situation(4). Potentiation of Wydase by uterone could be explained by increase of intradermal pressure caused by inflammation with ensuing wider diffusion of the enzyme and therefore a greater spread. The hyaluronate-inactive nature of uterone spreading factor is considered to be all the more likely since ovomucoid, a mucoprotein which shares many physical and chemical properties of uterone, causes in the rabbit an enhancement of spreading of india ink comparable to that observed with uterone. It may be noted here, as previously described by Hechter, that curves relating spreading to time are of 2 distinct types, one with rapid onset and S-shaped slope for the enzymatic spreading factor, the other typical for hyaluronate-inactive spreading factor with slowly ascending, progressively mounting slope. In addition, we noted that the area of ink spot retracts after 24 hours where inflammation and edema were instrumental in causing spreading. This type of curve was obtained repeatedly at various dosages of uterone and with several experiments done at different times by different observers. Uterone does not exert its spreading effect in dead skin, where inflammation and edema no longer occur but where enzymatic activity of injected hyaluronidase still is effective.

*Summary.* A spreading factor is contained in uterone which may be classified as a hyaluronate-inactive spreading factor exerting its effect through inflammation and edema. The nature of the component of uterone responsible for provoking inflammation remains to be determined and may well be a mucoprotein.

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## Question of Purity of Erythropoietic Factor Concentrates.[*] (24457)

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There is now much evidence for a circulating erythropoietic factor and attempts have been made to concentrate it. Active material in plasma of rabbits made anemic with phenylhydrazine can be precipitated by ammonium sulfate and has been associated with a mucoprotein fraction(1,2,3). We prepared ethanol precipitates from anemic plasma according to Grant *et al.*(4)[†] and found them similar to concentrates made with ammonium sulfate. The stimulating factor found in urine of certain anemic patients is probably of the same nature as that found in anemic animals(5). This report compares certain properties and potencies of various active preparations made in 4 different ways.

*Materials and methods.* One preparation was from human urine(6),[‡] the rest from plasma of anemic rabbits. These also were compared to a preparation made from plasma of normal rabbits. Starting material (PF) for some fractions was the filtrate obtained after boiling plasma at pH 5.5 for a few minutes(7). It contained only about 2% of plasma protein. We have shown that injections of unboiled plasma provoke immunological reactions which interfere with tests lasting 10 days or 2 weeks. After boiling there is no interference(3). The different preparations compared were: 1. PF from rabbits made anemic with phenylhydrazine was precipitated with 1.5 volumes of absolute ethanol. After 2 hours at 5°C the precipitate was removed in the centrifuge and an equal volume of absolute ethanol was added to supernatant. After 16 hours at 5°C it was centrifuged, the supernatant was poured off and the precipitate dried in a desiccator(4). About 25% of the protein and all erythropoietic activity of anemic PF was recovered in this fraction. In one case the yield was about 350 mg/l of

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[‡] We thank Dr. Van Dyke for a gift of this material.

TABLE I. Erythropoietic Activity of Various Preparations. Each assay was made with 6 mice or 4 rats, except where noted.

| | Assay | | | | | | |
|---|---|---|---|---|---|---|---|
| | Reticulocytes in normal mice after 3 doses | | $Fe^{59}$ uptake in fasted rats after 3 doses | | Hemoglobin in normal rats after 12 doses | | |
| | | | | | | Hemoglobin, g % | |
| Preparation and source | Dose | Reticulocytes, % | Dose | $Fe^{59}$, % | Dose | Day 1 | Day 15 |
| Saline | 1.0 ml | 2.1 | 2.0 ml | 5.1 | 5.0 ml | 14.7 | 14.2 |
| PF anemic rabbit plasma | 2.0 *† | 9.0 | 2.0 * | 32.0 | 5.6 * | 14.3 | 17.3 |
| 1 *Idem* | .05 mg<br>.25<br>2.0 | 4.0<br>7.1<br>11.0 | .06 mg<br>.24<br>.6 | 9.7<br>22.3<br>38.2 | | | |
| 2 " | .05<br>.18 | 5.4<br>10.2 | .5 | 14.2 | 2.16 mg | 14.2 | 17.1 |
| 3 " | .06<br>.18 | 6.5<br>8.6 | .93 | 21.5 | | | |
| 4 Human urine | .1<br>.5 | 4.8<br>8.5 | 1.0 | 25.6 | 2.5 | 14.5 | 16.9‡ |
| 5 *Idem* | | | 1.5 | 30.3 | | | |
| 6 Normal rabbit plasma | 1.3 | 2.1 | 9.0 | 6.1 | | | |

* 1 ml PF contains 1.0-1.5 mg protein. † Reduced in vol. ‡ 2 rats only.

plasma. Nos. 2 and 3 were made from a fraction of anemic rabbit plasma, insoluble between 50-80% saturation of ammonium sulfate(2,3). 2. Material insoluble between 50-80% saturation of ammonium sulfate was boiled and a fraction soluble in 70% ammonium sulfate prepared as previously described(2,3). The yield was 100-130 mg/l of plasma. 3. Material insoluble between 50-80% saturation of ammonium sulfate was dialysed, boiled at pH 5.5 in 0.9% NaCl, the pH changed to 7.5, concentrated till protein was 1.4 mg/ml and precipitated with ethanol as in No. 1. The precipitate contained 25% of the protein and all activity of the filtrate after boiling. The yield was 180 mg/l of plasma. 4. *Urinary preparation.* This was the residue from ultrafiltration of urine of a patient with paroxysmal nocturnal hemoglobinuria.‡ 5. The same as 4, boiled 5 minutes. 6. Made the same as 1, but from plasma of normal rabbits. About 25% of the protein of boiled plasma was recovered. Yield about 300 mg/l of plasma. Preparations 1, 2 and 3 from anemic rabbit plasma and 6 from normal plasma, all of which had been boiled in the presence of all or much of the plasma proteins, showed the same main band in paper electrophoresis (pH 8.6, 0.05M veronal) with a mobility slower than albumin and faster than $\alpha_1$-globulin. Preparations 4 and 5 from urine showed a main band between $\alpha_1$- and $\alpha_2$-globulin which after boiling was displaced to the origin. Activity of different fractions was tested by injecting them subcutaneously into mice or rats and by using the following assays: reticulocytes in normal mice after 3 daily injections(3), $Fe^{59}$ uptake in fasted rats after 3 daily injections(8), and circulating hemoglobin concentration after 2 weeks of injections(7).

*Results.* Preparations 1, 2, 3, 4, and 5 all have activity by whichever method was used to test them. The effective dose was of the same order of magnitude for all 5 in mouse reticulocyte and rat $Fe^{59}$ uptake assays (Table I).

In preparation 6 from normal rabbit plasma, we found no activity even at the high dose shown. This preparation can best be compared with 1, as they were made in the same way, yet 6 showed no activity at doses more than 20 or 30 times greater than 1.

*Discussion.* Isolation of such similar fractions of widely different effectiveness raises questions. The activity can be concentrated in a fraction containing highly soluble mucoproteins. It may be due to a special mucoprotein found only in anemic animals or to something not a mucoprotein. Because of the proteolytic destruction of activity(2,9) it is likely that it is of peptide nature or has such material as a necessary cofactor. Association with a different protein could account for the different electrophoretic behavior of the urinary material. Activity might be present in normal plasma but may not be separable by the methods effective with anemic plasma.

*Summary.* The bulk of erythropoietic activity of anemic plasma can be concentrated in a fraction which represents less than 0.5% of plasma proteins. Yet from the similarity in yield and electrophoretic behavior of the corresponding inactive fraction from normal plasma, it appears likely that the erythropoietic factor constitutes only a small portion of the present concentrates.

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## Lipoprotein Lipase Response in Laennec's Cirrhosis.* (24458)

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Since the observation that heparin injected *in vivo* cleared alimentary lipemia in dogs(1), much work has been done in elucidating the mechanism of this phenomenon. It has subsequently been demonstrated that heparin-activated clearing factor is a plasma lipoprotein lipase which acts upon the triglyceride moiety of plasma lipoproteins to hydrolyze them to free fatty acid and glycerol(2,3). These studies have evoked increased efforts to relate concentration of this lipase to disorders of lipid metabolism(4), particularly atherosclerosis(4,5). In each instance, emphasis has been placed upon a *decreased* clearing factor or lipoprotein lipase response. Methods used in measuring lipoprotein lipase response or concentration have varied widely. Consequently, until recently, no attempt had been made to determine mean clearing factor response for a given "normal" population. A standardized *in vitro* method has been developed for determining clearing factor concentration and has been applied to an agewise study of a presumably "normal" population (6). Inasmuch as no significant agewise regression was found in clearing factor response, a mean for this population was established. In this study, it was incidentally observed that patients with primary diagnoses of chronic alcoholism and/or Laennec's cirrhosis demonstrated a significantly *increased* clearing factor response(7). In view of the observation that there is a lesser incidence of atherosclerosis in patients diagnosed as Laennec's cirrhosis, more extensive studies have been made on a larger group of subjects reported herewith. In a small group of patients with recent myocardial infarction (presumptive evidence of coronary atherosclerosis) evaluated in the original study, no significant difference in clearing factor response was observed when tested against the normal popu-

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* This investigation was supported in part by a research grant from Nat. Heart Inst., P.H.S.

lation in the same age decades. The fact remains, however, that *none* of the "normal" subjects could be said to be absolutely free of atherosclerosis, particularly in ages 40 to 69 years studied. Consequently, for comparative purposes, and to evaluate their clearing factor response, patients with recent myocardial infarction have also been included in the group evaluated in this study.

*Material and methods. Subjects.* One hundred patients were selected without regard to age or sex from Medical Services of Cook County Hospital. Basis for selection was hepatic pathology of *any* etiology, jaundice, or recent myocardial infarction. One patient evaluated manifested the nephrotic syndrome. Twenty-nine patients demonstrated clinical and laboratory evidence of Laennec's cirrhosis. Heparin Sodium, 3 mg in 5 ml of isotonic saline[†] injected intravenously, was used as clearing factor (lipoprotein lipase) stimulus. *Lipoprotein lipase response.* The standardized *in vitro* Baker method for determination of clearing factor concentration(7) was employed. *Preparation of samples.* Fifteen milliliter control and 8-minute post-heparin blood samples were obtained from all subjects after overnight fast (at least 12 hours post-prandial). These samples were immediately mixed with dried sodium oxalate (1 mg/ml blood) and cooled to 0°C in ice bath. Plasma was separated by centrifugation at 0°C. It was subsequently kept at 0°C or frozen until used. *Substrate.* One % emulsion of coconut oil (Ediol)[‡] freshly prepared with distilled water was used as substrate. Three-tenths milliliter of this emulsion was added to and thoroughly mixed with a 4.5 ml aliquot of post-heparin plasma, which had been previously incubated for 10 minutes in water bath at 30° ± 1°C. This proportion, in general, gave an initial optical density reading between 0.6 and 0.7 against distilled water on Beckman Model DU Spectrophotometer, with microcuvettes (2.5 x 10 x 25 mm) at 6500 Å. The post-heparin plasma-fat emulsion mixture was kept at 30°C for one hour. During this time, optical densities were read every 5 minutes on 0.3 ml aliquots of the mixture. Optical densities of control plasma-fat emulsion mixtures were similarly determined. All samples were run in duplicate. *Clearing factor (lipoprotein lipase) concentration.* A graph of optical density versus time was then plotted for each sample. To this plot, a straight line was fitted at the section demonstrating greatest rate of change in optical density. The negative slope of this line, $k$ (decrease in optical density/hour), was taken to represent rate of clearing of post-heparin plasma, which, in turn, is directly proportional to lipoprotein lipase concentration. *Evaluation of hepatic status.* Comprehensive laboratory tests on aliquots of control blood samples included total and esterified cholesterol, serum bilirubin, icterus index, BSP, total proteins, albumin/globulin ratio, cephalin flocculation, thymol turbidity, gamma globulin turbidity, and alkaline phosphatase. Total serum lipids and lipid phosphorus were also determined on aliquots of control blood samples.

TABLE I. Increased Lipoprotein Lipase Response in Laennec's Cirrhosis.

| Diagnosis | No. of subjects | Mean $k$ | Range of $k$ |
|---|---|---|---|
| Laennec's cirrhosis | 29 | 1.16 | .37–2.25 |
| "Normal" | 84 | .74 | .13–1.40 |
| Infectious hepatitis | 11 | .58 | .00–2.12 |
| Recent myocardial infarction | 12 | .58 | .06–1.24 |
| Obstructive jaundice | 6 | .33 | .00– .86 |

$k$ is rate of decrease of optical density/hr. This is directly proportional to lipoprotein lipase (clearing factor) concentration in standardized *in vitro* assay method(6,7).

*Results.* These studies have confirmed the presence of an *increased* clearing factor or lipoprotein lipase response in Laennec's cirrhosis. Table I demonstrates the increased clearing factor or lipoprotein lipase response in patients with Laennec's cirrhosis as compared with "normal" subjects, patients with infectious hepatitis, recent myocardial infarction, and obstructive jaundice. As described in the original study(6,7), $k$ (decrease in optical density/hour) is directly proportional to clearing factor or lipoprotein lipase concentration. Clearing factor response was evaluated in terms of total serum lipids and lipid phosphorus, the former being generally low (below 600 mg/100 ml) in Laennec's cirrhosis. There appeared to be no correlation, however, between clearing factor response and levels of total serum lipids or lipid phosphorus in Laennec's cirrhosis. Serum total cholesterol generally ranged below 200 mg/100 ml. Per cent of serum cholesterol esters was almost uniformly low, being below 50% (below 75 mg/100 ml).

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[†] The authors wish to acknowledge the courtesy of Dr. E. A. Hawk of the Upjohn Co. in supplying heparin.

[‡] Ediol was kindly supplied by Mr. Robert E. Sullivan of Schenlabs Pharmaceuticals.

Clearing factor response and serum cholesterol/lipid phosphorus ratio in Laennec's cirrhosis are being more extensively investigated. When compared with Laennec's cirrhotics, a small group of patients with recent myocardial infarction demonstrated a higher cholesterol/lipid phosphorus ratio with a lower lipoprotein lipase response.

It has been observed that the hypoalbuminemia of the nephrotic syndrome markedly *decreases* clearing factor response(8) and that this decrease may be corrected by infusion of serum albumin. A single case of nephrotic syndrome (serum albumin 2.8 g/100 ml) demonstrated an almost negligible clearing factor response, which is in accordance with these observations. In patients with Laennec's cirrhosis, however, hypoalbuminemia produced *no depressant effect* upon clearing factor response, the greatest response being observed with serum albumin below 3.0 g/100 ml.

Lipoprotein lipase response in patients with other types of hepatic pathology such as infectious hepatitis, homologous serum jaundice, and obstructive jaundice has been variable. Observations on relatively small groups of patients to date permit no conclusions at this point. However, it may generally be stated that clearing factor response in these diseases does *not* appear to be elevated as in Laennec's cirrhosis.

*Discussion.* Perfusion experiments in rats have demonstrated that normal rat liver inactivates or attenuates clearing factor(9). It is, therefore, suggested that the *increased* clearing factor or lipoprotein lipase response in patients with Laennec's cirrhosis is related to their parenchymal liver cell damage. The intriguing possibility that parenchymal liver cell damage, either acute or chronic, may be reflected by concentration of heparin-activated lipoprotein lipase in plasma is currently being investigated. The possible inter-relationship between clearing factor response, cholesterol/lipid phosphorus ratio, lipid metabolism and atherosclerosis is also being explored.

*Summary.* 1. *Increased* lipoprotein lipase response has been observed in patients with Laennec's cirrhosis. 2. When compared with Laennec's cirrhotics, a small group of patients with recent myocardial infarction (presumptive evidence of coronary atherosclerosis) demonstrated a higher serum cholesterol/lipid phosphorus ratio with a lower lipoprotein lipase response. 3. Hypoalbuminemia in Laennec's cirrhosis produced *no depressant effect* on lipoprotein lipase response. 4. Other types of hepatic pathology and jaundice have demonstrated a variable lipoprotein lipase response, but *no* apparent *increase*.

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## Viscosity and Origin of Meconium in Meconium Ileus.* (24459)

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Meconium ileus, thought to be an early manifestation of the more generalized disease, mucoviscidosis, is a congenital disorder of the newborn in which abnormally viscous meconium obstructs the infant's intestinal tract. Until recently, little information has been available concerning the chemical nature of this abnormal meconium. In a comparative study of the composition of normal and abnormal meconium, Buchanan and Rapoport (1) found the latter to contain a greater amount of nitrogen, most of which was trichloroacetic acid (TCA) precipitable. While work was in progress in the authors' laboratory, Green *et al.*(2), reported results of paper electrophoretic analyses which demonstrated the presence of human serum albumin and what appear to be small amounts of alpha, beta, and gamma globulin in meconium from patients with meconium ileus. The present study was directed toward elucidation of the nature and origin of the viscous meconium in this disease.

*Materials and methods.* A sample of meconium was obtained, at surgery, from a 2-day-old infant suffering from meconium ileus. Soluble components of abnormal meconium were dissolved by stirring with water overnight at 5°C. The resulting suspension was centrifuged at 10,300 × *g* for 30 minutes. The supernatant solution (Sol. I) was clear and dark green. Addition of 10% TCA to Sol. I resulted in formation of a heavy precipitate.

*Results.* After dialysis of Sol. I against water, it was dialyzed against 2 l of pH 8.60, ionic strength 0.1, barbital buffer and subjected to electrophoresis with the Tiselius apparatus supplied by Frank Pearson Associates. Fig. 1 (Top) is a schlieren scanning diagram(3) of the boundaries after 172 min. at 5.23 v/cm. Planimetry of this pattern gives the following percentages of total pattern area, exclusive of salt boundary, for each component: A, 69.5%; B, 17.4%; and C, 13.1%. Semi-micro Kjeldahl analyses, performed on Sol. I and on supernatant solution obtained after precipitation of the protein in Sol. I with TCA, indicate 8.0% of dry weight of meconium to be water soluble, TCA precipitable nitrogen. If the protein present is 16% nitrogen by weight, it follows that 50% of dry weight of meconium is water soluble, TCA precipitable protein. In agreement with Green *et al.*(2), normal meconium was found to contain no TCA precipitable protein. Boundaries A, B, and C migrate in a fashion characteristic of albumin and alpha and beta globulin respectively in pH 8.60, ionic strength 0.1, barbital buffer. These results are in agreement with those reported elsewhere (2), but the present study does not demonstrate a boundary corresponding to gamma globulin.

Because it is known that the fetus swallows amniotic fluid at least as early as fifth month of intrauterine life(4), it was believed that proteins present in meconium may have originated from swallowed amniotic fluid. Accordingly, samples of amniotic fluid were obtained from apparently normal healthy females at parturition.[‡] A small amount of insoluble material was removed by centrifugation at 25,500 × *g* for 20 minutes. The supernatant solution was then concentrated 5 times by pervaporation. After dialysis against water and then against pH 8.60, ionic strength 0.1, barbital buffer, the concentrated amniotic fluid was analyzed by electrophoresis. Fig. 1 (Bottom) shows the pattern after 95 min. at 7.85 v/cm. Planimetry of this pattern gives the following component percentages: D, 64.8%; E, 22.4%; and F,

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[‡] We express our gratitude to Dept. of Obstetrics and Gynecology, Duke Univ. Medical Center for supplying amniotic fluid specimens.

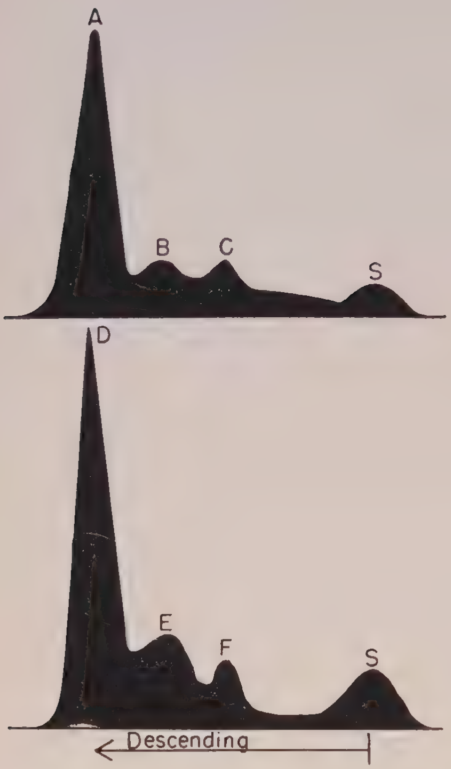

FIG. 1. (Top) Electrophoretic pattern of Sol. I. (Bottom) Electrophoretic pattern of amniotic fluid. *S* denotes salt boundary.

12.9%. Similar results have been reported elsewhere(5).

The 2 proteins forming boundaries A and D were purified from Sol. I and amniotic fluid by the technic of Schwert(6). The identity of these proteins with human serum albumin was indicated by their similar electrophoretic mobilities and sedimentaton constants and was confirmed by Ouchterlony technic(7) of double diffusion in agar employing solutions of these proteins and rabbit antibody to human serum albumin.

To determine the effect of added protein on plastic properties of normal meconium, a pooled sample of meconium from 2 apparently normal newborn infants was lyophilized and dry weight determined. The lyophilized meconium was then divided into 2 portions. To one was added crystalline human serum albumin (Cohn Fraction V) such that its concentration in normal meconium was that found in abnormal meconium. The other portion was retained as a control. The appropriate volume of water, based upon dry weight determinations of the abnormal meconium, was added to each. After thorough mixing, the 2 samples were compared with each other, and with samples of normal untreated meconium and with meconium from the patient with meconium ileus. The lyophilized sample with added albumin was much more viscous and tenacious than either control or untreated normal meconium, and appeared identical with meconium from the patient with meconium ileus.

*Discussion.* The theory that an abnormally viscous mucoprotein may be produced in patients with meconium ileus has been invoked to explain the viscous nature of meconium found in this disease(8). In view of the effect of albumin upon normal meconium, and that pancreatic digestive enzymes are either diminished or totally absent in patients with this disease(8), such an hypothesis would seem unnecessary. From present results, it appears that amniotic fluid is the source of proteins in abnormal meconium. Presumably proteins continually ingested in amniotic fluid are unable to be hydrolyzed, but are mixed with components of normal meconium and are concentrated by intestinal absorption of water to produce meconium characteristic of meconium ileus.

*Summary.* 1) Meconium from a patient with meconium ileus has been studied electrophoretically, revealing presence of albumin and of smaller amounts of other proteins with mobilities corresponding to $\alpha$ and $\beta$ globulin. The identity of albumin was established by immunochemical and physical methods. Semimicro Kjeldahl analyses of the abnormal meconium indicate 50.2% of dry weight of meconium to be water soluble, TCA-precipitable protein. Addition of human serum albumin to lyophilized pooled samples of meconium from normal infants results in meconium grossly indistinguishable from abnormal meconium. 2) The electrophoretic pattern of human amniotic fluid is identical with that of meconium ileus meconium and it is suggested that amniotic fluid is the source of protein present in meconium from patients with meconium ileus.

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# Effect of Sonic Energy, Ultracentrifugation, and Phenol on *Neisseria gonorrhoeae* Endotoxin.* (24460)

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For certain endotoxin-antigen studies the natural cell-free complex is desired. For other studies the smallest active unit of the original biologic complex is necessary. The present investigations deal with both types of products.

*Preparation of soluble nucleoprotein-bound endotoxin by physical methods.* Cultivation of *N. gonorrhoeae*† has been previously described(1,2). The gonococcus cells were washed twice with 4 volumes of saline and once with 4 volumes of distilled water. A supply of such cells was kept frozen at –20°C. Washed cells contained 10-12% solids. Three g portions of washed cells were suspended in 47 ml portions of distilled water and subjected to sonic disintegration in Raytheon 10-kc sonic oscillator at 8°C for 60 minutes at maximum intensity. Cell debris, 8.8-9.0%, on dry cell basis, was removed by centrifuging in cold room in Servall angle centrifuge at 3,600 g for 15 minutes. The clear supernatant was placed in cellophane bags and dialyzed for 20 hours with continuous stirring against distilled water. The supernatant, after dialysis, contained 90% of solids originally present in the gonococcus cells (5-6 mg protein/ml). Analysis indicated that this material consisted mainly of nucleo-proteins. Such supernatants were stored at –20°C for several months without loss of toxicity. Precipitates which occasionally formed after thawing contained part of the endotoxin, and were put back in solution by brief sonic treatment. $LD_{50}$ values on the supernatant are listed in Table I. Twenty-five to 50 CFl male mice, weighing 16-18 g, and 5 different endotoxin concentrations were used in the assays(2). Endotoxin solutions were administered intraperitoneally. The animals were observed 5 days. Karber's(3) procedure was employed for calculating $LD_{50}$ values and interpreting their statistical meaning. From sonic treated supernatant a stable endotoxin-containing acetone powder was prepared as follows: a small quantity of sodium chloride was added to the supernatant and

TABLE I. Endotoxin Activities of Fractions Obtained by Sonic Treatment and Ultracentrifuging.

| Type of fraction | $LD_{50}$ value, mg |
|---|---|
| Supernatant at 3,600 g after sonic treatment | 1.6 |
| Sediment at 26,500 g in 30 min. | 1.1 |
| *Idem* 60 ” | 1.3 |
| Supernatant at 26,500 g in 60 min. | 1.4 |
| Acetone powder from same supernatant after removal of sediment | 1.5 |
| Acetone powder, cells extracted at pH 13 | 2.3 |
| Protein-free endotoxin | .8 |

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† The gonococcus strain employed was isolated from male patient by Dr. James D. Thayer of this laboratory.

the endotoxin was precipitated, together with nucleoproteins and a small quantity of other proteins, by addition of 3 volumes of cold acetone. The precipitate was removed by centrifugation and dried over sodium hydroxide *in vacuo*. The yield of dry material was 90% on the basis of dry gonococcus cells. Endotoxin precipitates are only very slightly soluble in water. The dry powder was readily put in solution, however, by suspension in distilled water and brief sonic treatment. An aliquot of the supernatant obtained after sonic treatment of the gonococcus cells, and dialyzed as described above, was centrifuged in the Spinco Model L ultracentrifuge at 26,500 g for 60 minutes at –10°C. Thirty to 35% of the endotoxin sedimented under these conditions. The sediment was put in solution by suspension in distilled water and brief (15 minutes) sonic treatment. When thawed supernatant was used, sedimentation was much heavier (up to 75%). Occasionally endotoxin-containing material precipitated on freezing. This precipitate was not removed before ultracentrifuging. An increase in speed up to 100,000 g did not result in heavier sedimentation. Endotoxin is firmly bound to nucleoproteins and speeds up to 100,000 g did not result in sediments or supernatants of significantly different toxicities (Table I). The endotoxin remaining in the supernatant after centrifugation was removed by addition of small quantity of sodium chloride and 3 volumes of acetone.

*Preparation of protein-free endotoxin.* Here a phenol-water mixture, first employed by Palmer and Gerlough(4) in preparation of antigenic-substances from other organisms, was used. Fifty g of washed gonococcus cells were suspended in 45 ml of distilled water. The cell suspension was poured into Waring blendor containing a mixture of 110 g phenol and 67 ml distilled water. The contents of the Waring blendor were stirred for 8 min. The temperature of mixture was 25°C at the beginning, and 45° at end of stirring. Then the mixture was cooled to 20°C and centrifuged in cold room in Servall angle centrifuge at 3,600 g. The clear supernatant (aqueous layer) was placed in cellophane bags and dialyzed for 24 hours at 4° against 14 l of distilled water. The latter was stirred continuously and changed occasionally. The lower (phenol) layer containing the proteins was discarded. A small quantity of sodium chloride was added to the dialyzed supernatant. The endotoxin was precipitated by the addition of 1.5 volumes of cold acetone. The precipitate was washed once with cold acetone and once with cold ether, and dried over sodium hydroxide *in vacuo*. The yield of dry material was 350 mg.

*Composition.* This preparation had an $LD_{50}$ of 0.8 mg (Table I). It contained 52% total saccharide, as measured by Dische's(5) indol method, 53% nucleic acid, and 8.5% nitrogen. It contained 0.2% serum albumin equivalent (probably polypeptide), as found by Folin-Ciocalteu procedure, and 8% lipid. The latter was determined as follows: 200 mg of protein-free material was dissolved in 39 ml of distilled water, 1.1 ml of conc. sulfuric acid added and the mixture boiled with refluxing for 1.5 hours. The cooled hydrolysate was shaken with chloroform and centrifuged. Chloroform phase was removed, filtered and evaporated to yield 16 mg of wax-like lipid. We were unable to remove from the protein-free endotoxin the nucleic acid component with ion-exchangers or by fractionation with acetone or ethanol. Uranium acetate precipitated both the polysaccharide component and the NA moiety. *Absorption curves.* In Fig. 1 is seen the ultraviolet absorption curve of the natural nucleoprotein complex endotoxin, labeled NP, together with the absorption curves of yeast nucleic acid (YNA), solid line, and the protein-free endotoxin (PF). All show a peak at 260 mμ. NP shows an absorption curve typical of plant nucleoproteins. The PF absorption curve indicates removal of protein from the natural product. Only slightly different products were obtained when hydrolysis with phenol water was carried out at lower temperatures, or by applying mechanical mixing instead of extraction in the Waring blendor, or by using phenol-water mixtures of different proportions.

*Summary.* 1) Sonic disintegration puts about 90% gonococcal endotoxin into a soluble state as contrasted with 50% yields by earlier methods. Most fractions obtained by

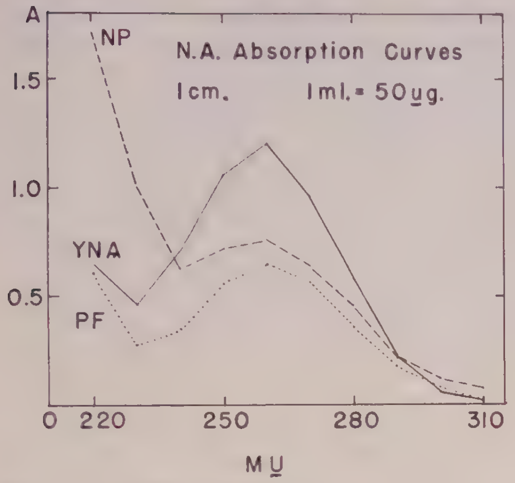

FIG. 1. Absorption spectra. NP = natural nucleoprotein complex; YNA = yeast nucleic acid; PF = protein-free endotoxin.

this physical procedure did not differ statistically among themselves in toxicity, but all of them were invariably more toxic than the product obtained by extraction of gonococcal cells at an alkaline pH. It is believed these methods will be useful in biological studies where cell-free, chemically unchanged fractions are desired. 2) Phenol-water mixtures are suitable for removal of the protein component from nucleoprotein complex of *N. gonorrhoeae* endotoxin. The protein-free endotoxin contains mainly polysaccharide and NA. The dried product is more toxic than those obtained by other methods. It dissolves readily in water resulting in a slightly opalescent solution. This fraction should be useful in studies where portein-free material is desired.

Addendum: While this paper was in press we were able to remove all of the NA from the endotoxin. The method employed, chemical nature and other properties of the product will be described in subsequent publication.

We wish to thank Mr. Harold Russell for skillful technical assistance.

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# Effect of 4-Amino Folic Acid Antagonists on Biological Acetylations.* (24461)

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Previous studies have shown that p-aminosalicylic acid (PAS) inhibits acetylation of isoniazid (INH), and produces a marked increase in plasma levels of INH when the 2 drugs are administered in combination(1). The synergistic effect of the INH-PAS combination observed in tuberculosis therapy has been attributed, in part, to inhibition of INH acetylation by PAS(2). It is conceivable that similar events may occur when multiple antimetabolite combinations are employed in cancer chemotherapy. Thus, apart from the anticancer effect contributed by each member of the combination by virtue of its antimetabolite activity, secondary factors may be present to further increase the over-all effect(3). For example: 6-amino-nicotinamide (6-AN), a carcinostatic antimetabolite (4-7) is a competitive inhibitor of arylamine acetylation, and is itself acetylated in rabbits(1,8). Administration of this substance in combination with a second antimetabolite having similar properties with respect to acetylation could give rise to increased plasma and cellular concentrations of either substance, due to inhibition of acetylation. The end result could be

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TABLE I. Inhibition of Sulfanilamide (SA) and Isoniazid (INH) Acetylation in Pigeon Liver Extracts.

| Inhibitor | Inhibitor conc., M/l × $10^{-5}$ | Inhibition, % SA | Inhibition, % INH |
|---|---|---|---|
| A-methopterin (4-amino-$N^{10}$-methyl-PGA) | 5 | 99 | |
| | 2 | 73 | 56 |
| | 1 | 62 | |
| Aminopterin (4-amino-PGA) | 25 | 89 | |
| | 5 | 58 | |
| | 4 | | 50 |
| Folic acid (PGA) | 100 | 0 | 3 |
| 2,4-Diaminopteridine | " | 0 | 8 |
| 2,4-Diamino-6,7-diphenylpteridine | " | 50 | 50 |
| Para-aminobenzoylglutamic acid (PABGA) | " | | 39 |

Incubation mixture: Potassium phosphate buffer, pH 7.4, .02 M; potassium acetate, .02 M; potassium citrate, .02 M; sodium ATP, .004 M; SA or INH, 8 × $10^{-4}$ M; pigeon liver extract, 1 ml (equivalent to 60 mg of acetone powder); acetylation inhibitors, as indicated; total volume, 3 ml. Incubation for 30 min. at 37°C.

augmented carcinostasis, increased host toxicity, or both. It was therefore of interest to test the effects on arylamine acetylation of various carcinostatic antimetabolites which may be employed in combination with 6-AN. In the course of these investigations it was found that A-methopterin and aminopterin markedly inhibited acetylation of sulfanilamide and INH in a non-competitive manner. This inhibition was due, in part, to an effect on acetate activation.

*Materials and methods.* Procedures employed in the sulfanilamide and INH acetylation studies were as previously described(8,1). Acetyl-Coenzyme A formation in pigeon liver extracts was measured by the method of Chou and Lipmann(9) and hydroxamate formation according to Lipmann and Tuttle(10). The methods for *in vivo* acetylation studies were as previously described(11). Aminopterin and A-methopterin (Methotrexate) were generously supplied by Dr. J. M. Ruegsegger of Lederle Lab. Div., Am. Cyanamid Co. These substances were used without further purification. 2,4-Diaminopteridine and 2,4-diamino-6,7-diphenylpteridine were obtained through the courtesy of Dr. E. Rohrman, The Eli Lilly Research Laboratories, Indianapolis, Ind.

*Results.* A-methopterin and aminopterin at $10^{-3}$ M inhibited completely acetylation of sulfanilamide and INH. From Table I it can be seen that the concentration required for 50% inhibition was, in the case of A-methopterin, less than $10^{-5}$ M, and for aminopterin, 5 x $10^{-5}$ M. Folic acid (PGA) was not inhibitory at $10^{-3}$ M, and also failed to reverse the inhibition caused by A-methopterin and aminopterin. Furthermore, acetylation was inhibited only 50% by $10^{-3}$ M 2,4-diamino-6,7-diphenylpteridine and not at all by $10^{-3}$ M 2,4-diaminopteridine. These results indicated that the 4-amino group of A-methopterin and aminopterin was directly involved in the inhibitory effect, and that the 2-amino group, which is also present in folic acid, was inactive. It was also apparent that the pteridine moiety, alone, did not account for the whole inhibitory effect. No diazotizable amine was produced when A-methopterin or aminopterin was incubated with the complete acetylating system in absence of sulfanilamide, thus indicating that the observed inhibition was not partly due to an artifact arising from cleavage of the molecule with consequent release of para-aminobenzoylglutamate (PABGA). Moreover, the latter at $10^{-3}$ M inhibited INH acetylation by only 39% (Table I). Para-aminobenzoic acid (PABA) at the same concentration was previously shown to inhibit INH acetylation by 41%(1).

The nature of the inhibition by A-methopterin was investigated by using varying concentrations of sulfanilamide and A-methopterin. The results, plotted by the method of Lineweaver and Burk(12) indicated that the inhibition was non-competitive (Fig. 1). Attempts to reverse the inhibition by addition of cysteine or excess Coenzyme-A were unsuccessful. Citrovorum factor, not being readily available, was not tried.

The hydroxamic acid method of Chou and Lipmann(9) was used to study the effect of A-methopterin on activation of acetate to acetyl-S-CoA. As shown in Table II, acetate activation was inhibited only 33-36% by $10^{-4}$ M A-methopterin, at which concentration complete inhibition of sulfanilamide acetylation occurs. It is thus apparent that inhibi-

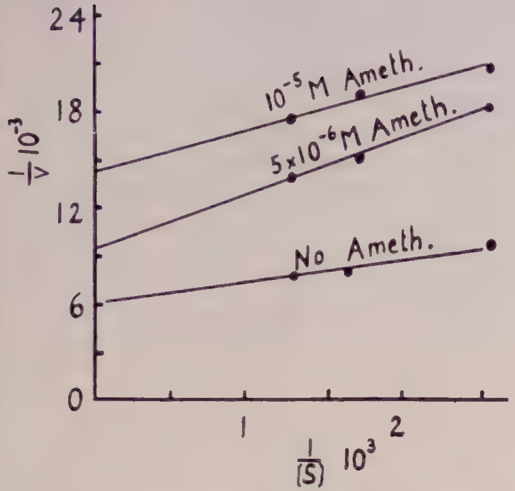

FIG. 1. Non-competitive inhibition of sulfanilamide acetylation by A-methopterin (Ameth.). Velocity (V) expressed as $\mu$g of sulfanilamide acetylated in 30 min., and sulfanilamide concentration (S) as molarity. Other conditions are given under "*Materials and methods.*"

tion of acetate activation can account for but a part of the total inhibitory effect of A-methopterin on acetylation.

The experimental data presented in Table III suggest that A-methopterin also modifies the acetylation of sulfanilamide *in vivo.* When A-methopterin was administered to rabbits conjointly with sulfanilamide mean blood levels of free sulfanilamide were markedly higher, at 2, 4, and 6-hour intervals after dosage, than when sulfanilamide alone was given. Furthermore, there was a concomitant decrease in plasma levels of acetylated sulfanilamide. In Exp. I, Table III, the dose of A-methopterin (25 mg/kg) has been calculated as the equivalent of the concentration required for 50% inhibition *in vitro.* Fountain *et al.*(13) have reported that administration of A-methopterin to mice resulted in retention of the compound in the liver for 3 weeks. To test the possibility that A-methopterin might be similarly retained by rabbit liver and lead to prolonged inhibition of acetylating ability, a rabbit was given 50 mg/kg of A-methopterin 18 hours prior to a single dose of 200 mg/kg of sulfanilamide. No impairment of acetylation was observed, and the high dose of A-methopterin appeared to have no deleterious effect on the rabbit. It is conceivable that resistance of the rabbit to the toxic effects of folic acid antagonists(14), may be due to ability to acetylate the 4-amino group of these compounds. However, no evidence for this has yet been obtained.

*Discussion.* The 4-amino derivatives of folic acid have been shown to inhibit conversion of folic acid to citrovorum factor (CF), and to interfere with utilization of preformed CF(15). The toxic effects of these analogues are blocked by CF administration, or prior injection of large amounts of folic acid. The present studies indicate that the 4-amino folic acid analogues are potent non-competitive inhibitors of arylamine acetylation, an activity which is apparently quite unrelated to the antimetabolite function of these compounds.

TABLE II. Effect of A-methopterin on Acetate Activation in Pigeon Liver Extract.

| Exp. | A-methopterin | Acethydroxamate formed ($\mu$moles) | % inhibition |
|---|---|---|---|
| 1 | 0 | 3.68* | |
| | $10^{-4}$ M | 2.47 | 33 |
| 2 | 0 | 3.62 | |
| | $10^{-4}$ M | 2.34 | 36 |

Incubation mixture: As in Table I, except that 0.5 M hydroxylamine, pH 7.4, replaced SA or INH; and 1 ml of pigeon liver extract (equiv. to 120 mg of acetone powder) was used. Incubation, 1 hr at 37°C.

* When acetate was omitted 0.53 $\mu$moles of acethydroxamate were formed.

TABLE III. Effect of A-methopterin on Plasma Levels of Sulfanilamide in Rabbits.

| | | Drug dose (mg/kg)* | | Sulfanilamide in blood (mg/100 ml)† | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Hr after dosing | | | | | |
| | | | | 2 | | 4 | | 6 | |
| Exp. | No. of rabbits | Sulfa. | A-meth. | Free | Acet. | Free | Acet. | Free | Acet. |
| 1 | 3 | 400 | | 14.0 | 10.6 | 9.4 | 8.1 | 7.2 | 8.1 |
| | | " | 25 | 23.1 | 6.8 | 15.4 | 6.1 | 10.4 | 6.2 |
| 2 | 3 | 200 | | 5.0 | 3.7 | 3.8 | 5.2 | 2.4 | 9.3 |
| | | " | 35 | 7.8 | 3.6 | 6.3 | 4.7 | 4.0 | 5.4 |

* Drugs were given orally as a suspension in water.

† Mean values.

The mechanism of action is not fully known. The inhibitory effect on acetyl-CoA formation (Table II) was too weak to account for inhibition of sulfanilamide acetylation by A-methopterin *in vitro*. Furthermore, it is unlikely that an effect on acetate activation is responsible for inhibition of sulfanilamide acetylation in rabbits, since acetyl-CoA can be generated at normal rates from other metabolic precursors. It is possible however that these substances may displace acetyl-CoA from the acetylating enzyme. A further possibility is that inhibition of acetylation may be only one instance of a more generalized phenomenon. Fountain *et al.*(13) observed that, following a parenteral dose of A-methopterin in mice, a more or less constant amount of the drug was retained in the liver for at least 3 weeks. The authors suggested that the retained drug was probably concentrated in the enzymes associated with folic acid and CF. It is now apparent that the folic acid antagonists have a high affinity for enzymes associated with arylamine acetylation, and probably others.

It is not possible to say whether, at the dosage levels presently employed therapeutically, sufficient concentrations of the folic acid antagonists can accumulate in the cell to inhibit acetylation processes. Such a possibility should not be lost sight of, particularly in the event that aminopterin or A-methopterin is administered in combination with a drug known to be acetylatable, such as 6-aminonicotinamide. Indeed, one might expect that simultaneous administration of 6-aminonicotinamide and a 4-amino folic acid antagonist would result in carcinostatic potentiation.

*Summary.* 1) Acetylation of sulfanilamide and of isoniazid in pigeon liver extracts was markedly inhibited (non-competitively) by 4-amino analogues of folic acid. A-methopterin ($10^{-5}$ M) and aminopterin ($5 \times 10^{-5}$ M) inhibited acetylation by 62 and 58%, respectively. Folic acid ($10^{-3}$ M) was not inhibitory, and failed to reverse the inhibition by A-methopterin. 2,4-Diamino-6,7-diphenylpteridine ($10^{-3}$ M) gave 50% inhibition of acetylation and 2,4-diaminopteridine ($10^{-3}$ M) was inactive. A-methopterin, administered to rabbits conjointly with sulfanilamide, produced a marked increase in plasma level of free sulfanilamide, and a concomitant decrease in acetyl-sulfanilamide. 2) The possible significance of these results with regard to combination chemotherapy of cancer was discussed.

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# Autoradiographic Studies of Leukocyte Formation.* (24462)

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Results of recent investigations by Hamilton(1), Ottesen(2), Osgood(3), and Resegotti(4) have shown a considerable difference between life spans of granulocytes and lymphocytes in mammals. Their studies indicate a life on the order of several days for granulocytes and several weeks for the majority of lymphocytes. The classical technic of thoracic lymph duct cannulation(5), however, has shown in several mammals that enough lymphocytes enter the circulation from thoracic lymph alone to replace the entire blood lymphocyte pool several times daily. Comparable to this is a life span for the granulocyte of 23 minutes from parabiotic rat experiments of Van Dyke and Huff(6). Classical transfusion experiments as well as recent transfusion experiments with tagged cells have also indicated rapid disappearance of injected leukocytes(7). The apparent conflict may be resolved by employing a label for newly formed leukocytes permanent for the life of the cells, and simultaneously studying cells in hematopoietic tissues and circulation. The autoradiographic technic used here enables a measurement to be made of the activity of individual cells which have incorporated a radioactive DNA precursor. We report data based on analyses of labeling seen in different classes of white cells in blood and blood-forming tissues and analysis of their separate life spans. This is not possible by the biochemical methods employed in the past.

*Methods.* Adenine $C^{14}$ is a convenient label for cells synthesizing new DNA during an interval at start of experiment(8). Adenine is quickly taken up in cells in the form of nucleotides and at some later time is synthesized into both RNA and DNA. It is now generally agreed that once DNA has been synthesized from nucleotides, it remains and is stable for the life of the cell, or until it is segregated at the next division. Active DNA in a cell is then a permanent label for the cell. 8.5 $\mu$C adenine 8-$C^{14}$ (2.2 mC/mm) were injected intraperitoneally into 10-11 week-old male A strain mice weighing 23 g. The animals were sacrificed at regular intervals following injection and smears were made from various tissues—tail blood, thymus, lymph nodes, and femoral marrow. Smears were fixed in absolute methanol for 1 hour, followed by distilled water 1 hour to remove unpolymerized nucleotides. Duplicate smears were also digested with ribonuclease to remove RNA. Then essentially the only remaining activity is due to adenine incorporated into DNA. Smears were next prepared with English Kodak Ltd. AR-10 autoradiographic stripping film(9) and exposed 30 days. Then the slides were photographically processed and subsequently stained through the film with Giemsa stain. Grain counts were made over individual cells and results recorded for a minimum of 1000 cells/slide. A cell was considered labeled if it had more than 2 grains, which was above the background. Lymphocytes of diameter $<8$ $\mu$ were classified as small lymphocytes, 8 to 10 $\mu$ as medium lymphocytes, and $>10$ $\mu$ as large lymphocytes. Band neutrophils in the marrow were distinguished from mature forms by the smooth ring shaped nucleus. When the nucleus showed some folding it was considered to be a mature neutrophil.

*Results.* Fig. 1 shows the strikingly different courses of adenine incorporation into DNA in the 3 major classes of white cells in peripheral blood. Labeled neutrophils rise sharply from the second to fourth day after injection, by which time more than half the cells are labeled. This is followed by a rapid decline. Labeled small lymphocytes, on the other hand, form a plateau at 2-3% that lasts at least 8 days. This indicates that most of the small lymphocytes were formed before start of experiment in contrast to neutrophils. Correspondingly, the life of the small lymphocyte is long compared to the neutrophil. In contrast to the small lymphocytes a high per-

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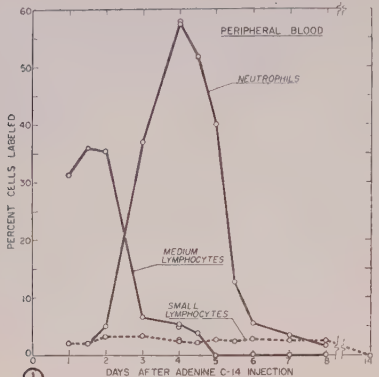
* This work was supported by U. S. Atomic Energy Comm.



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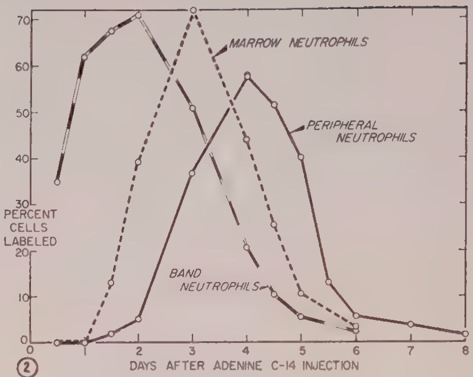

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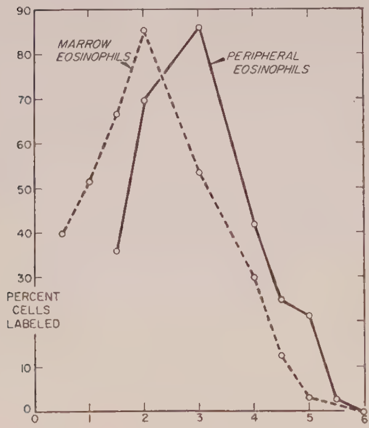

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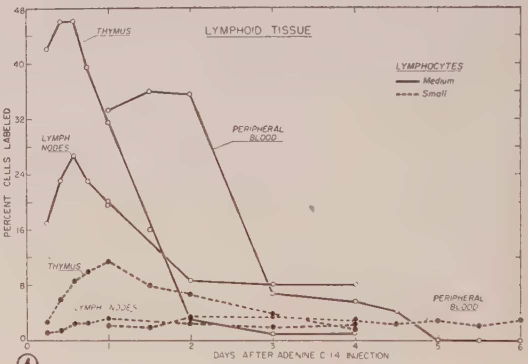

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FIG. 1. Percent of circulating leukocytes with labeled DNA after a single inj. of adenine C-14.

FIG. 2. Percent labeled neutrophils seen in marrow and peripheral blood after a single inj. of adenine C-14.

FIG. 3. Percent eosinophils labeled after a single inj. of adenine C-14.

FIG. 4. Percent DNA labeled lymphocytes seen in lymphoid tissues and peripheral blood after a single inj. of adenine C-14.

centage of labeled medium and large lymphocytes were found at 1 and 2 days and disappeared by fifth day.

To interpret the curve for neutrophils in peripheral blood, data must be known on the immediate precursors in marrow. Fig. 2 shows percentage labeled cells for the last 2 maturation stages of neutrophils—the band and mature neutrophils in the marrow—together with the peripheral neutrophils. It should be noted that these values are from bone marrow smears not extracted with ribonuclease since this treatment would make marrow cell identification impossible. Peripheral neutrophils contain little RNA label, however, since RNase treatment does not measurably lower the activity of this class. This may be true of mature marrow neutrophils since they have lost the staining property for RNA in the cytoplasm.

Data of Fig. 2 show that labeled cells do not enter the mature marrow neutrophil pool until about 1 day, suggesting that this is the minimum time required for differentiation of mature neutrophils from the last division stage. The preponderance of band cell forms over earlier stages of differentiation as well as the initial portion of the band neutrophil curve, indicate that almost a whole day is spent in the band cell stage. This may be taken as a maximum for band cell maturation time.

These observations may be used to approximate relative pool sizes and turnover times of marrow and peripheral neutrophils. Consideration of the time relation between labeling, seen in band and mature marrow neutrophil classes, indicates that release of mature neutrophils to circulation tends to be orderly rather than random. Similar consideration of the relation between labeling seen in mature marrow neutrophils and peripheral neutrophils indicates that neutrophils disappear from the periphery at the end of a finite life span rather than randomly. The relation between the 2 classes has been approached by a comparison of experimental blood values with theoretically predicted values derived from labeling, in mature marrow neutrophils which are the immediate precursors to the peripheral neutrophils. The best fit of predicted values to experimental values has been obtained with a finite time of ⅔ day for duration of mature cells in marrow, followed by 1 to 1⅓ day life span in the periphery.

Such computations for levels of labeled cells agree closely with the experimental, whether we choose population of all cells labeled, or autoradiographically more definitive population of cells having more than 10 grains. These 2 independent calculations do not exclude the probability of a small degree of randomness in release of cells from each pool. The presence of a few band cells in the circulation indicates that some cells are randomly released from the marrow.

The steady state requires the flux of cells through each pool to be constant and equal. Therefore, if $V_1$ and $V_2$ are sizes of band and mature marrow neutrophil pools respectively $\frac{V_1}{T_1} = \frac{V_2}{⅔\ \text{day}}$ where $T_1$ is maturation time of band cells. The marrow differential indicates that $V_2/V_1 = 0.8$. $T_1$ is then about 20 hours in good agreement with the value derived from labeling.

If $V_3$ is the size of the peripheral neutrophil pool, then applying the steady state condition $\frac{V_2}{⅔\ \text{day}} = \frac{V_3}{1\text{ to }1⅓\ \text{day}}$. Therefore, $V_3/V_2$, the ratio of peripheral to marrow neutrophils is between 1.5 and 2. Since the circulating vascular pool is known to be quite small compared to the mature marrow pool, it must be concluded that peripheral neutrophils spend a large part of their time extravascularly. This agrees with conclusions published recently by several authors (for review see Patt, 10).

Approximate data on eosinophils are presented in Fig. 3. Eosinophils regularly comprised only 7-10% of granulocytes so that each point represents only 30 to 60 cells counted. This has precluded any quantitation such as was attempted with neutrophils; however, it is clear that labeled eosinophils appeared and disappeared more rapidly than did neutrophils in the circulation.

Data on events in lymphoid tissues are presented in Fig. 4. The most striking finding is the high activity of mouse thymus compared to lymph nodes. By the third day, however, essentially all active cells have disappeared from the thymus, indicating that turnover time for thymus cells is in the range of 2 to 3 days.

Lymph nodes reach a much shallower peak and retain a comparatively large proportion of active cells for at least 4 days. This suggests that lymph nodes have a long turnover time compared to thymus. These turnover times are in good agreement with our biochemical estimates from $P^{32}$ incorporation into DNA—2 days for thymus and 1 week for lymph nodes in A strain mice.

Labeled medium lymphocytes of nodes, thymus, and blood reach a higher peak earlier in time than that of small lymphocytes in these tissues. This is consistent with the hypothesis of a precursor-product relation between these 2 classes of cells. In addition, early label in the small lymphocyte class could be due to a few small lymphocytes preparing for division in accordance with the scheme of Sainte-Marie and Leblond(11).

In peripheral blood labeled small lymphocytes plateau at 2-3% for the first 8 days and disappeared by the fourteenth day. Small lymphocytes therefore live for at least 8 days. A large part of this time is probably spent in the organized or diffuse lymphoid tissue rather than in the circulation. The simplest interpretation of the data appears to be that cells released from the nodes into the circulation represent a random sample of cells present in the nodes at any time. This would reconcile the apparent discrepancy between the short circulating life calculated from thoracic duct outputs and the long life obtained from DNA labeling. To clarify this further the present experiments are being repeated using tritiated thymidine as DNA precursor. To date excellent agreement has been obtained between adenine and thymidine data. Thoracic duct lymphocytes are labeled to a degree consistent with the above concept (Schooley, Bryant, Kelly, to be published).

*Summary.* 1. Twenty-four hours are required for maturation of the neutrophil from the last division stage in the marrow. 2. The data are best explained by a finite life of ⅔ day for mature neutrophils in marrow, followed by a peripheral life span of 1 to 1⅓ days. 3. Lymphocytes fall into two classes—medium and large cells, which are rapidly renewed, and small cells, which live at least a week.

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# Effect of Endotoxin on Vascular Reactivity to Epinephrine in the Perfused Dog Forelimb and Lung.* (24463)

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Recent publications have indicated that endotoxin may have a direct effect on blood vessels, altering their reactivity to epinephrine (1-4). Changes in reactivity to epinephrine were reported to occur in blood vessels of the isolated perfused rabbit ear and in the mesoappendix of the rat following administration of small doses of endotoxin. Injections of larger doses resulted in vascular hyperreactivity to epinephrine followed by hyporeactivity. Arterioles and venules became completely refractory to epinephrine, while increased reactivity persisted in larger arteries and veins. The work of Zweifach, Nagler and Thomas(1) suggested very large changes in vascular reactivity after endotoxin in the rat mesentery, ranging from 100 times less, to 700 times greater than the pre-endotoxin response. It was postulated that changes in reactivity to epinephrine might be responsible for damaging effects of endotoxin on the vascular systems of intact animals and could account for certain resemblances between endotoxin reactions and "intoxication" by epinephrine(2). It was suggested that the end result of altered reactivity to epinephrine was pooling of blood in distended capillaries and venules(1). A number of vascular responses to endotoxin have been reported from this laboratory(5-8). Constrictions of blood vessels in a variety of organs have been observed

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* We are indebted to Dr. W. W. Spink, Univ. of Minnesota Medical School, for supply of endotoxin. This research was supported by grants from Minnesota Heart Assn and U.S.P.H.S.

Technical aid was helpfully given by Mr. Henry Ballin.

† Research Fellow of Life Insurance Medical Research Fund.

‡ Markle Scholar in Medical Sciences, 1952-1957.

§ Research Fellow of Am. Heart Assn.

*in vivo* and in the isolated perfused state(9-12). Changes in vascular tone after endotoxin have been suggested by decrease in peripheral resistance in eviscerated dogs(13). In view of findings of Zweifach and others it was believed that the lethal effect of endotoxin on the dog might be partially explained on the basis of altered vascular reactivity to epinephrine. A series of experiments was therefore carried out on isolated, perfused lung and forelimb of the dog. Results from these studies fail to indicate significant degree of altered response to intra-arterially injected epinephrine after administration of endotoxin to the isolated perfused dog forelimb and lung.

*Methods.* Adult mongrel dogs, anesthetized with sodium pentobarbital, 30 mg/kg body weight, were used. Freshly drawn heparinized homologous blood (5 mg heparin/100 cc blood) was obtained from donor animals. Perfusion studies included the following preparations: (a) Pump-perfused lungs, and pump-lung perfusions with an intact dog providing blood for the lung inflow circuit; (b) pump-lung perfused forelimbs, and pump-perfused forelimbs with intact dog providing arterial blood for the limb. In the first experiments, lungs were removed from heparinized dogs and perfused at constant flow with a sigmamotor pump as previously described (12). Lungs were perfused at highest flows compatible with isogravimetric state and were continuously weighed by a strain gauge weighing device(12). Perfusion pressure was measured in the pulmonary artery and a polyethylene catheter was advanced in retrograde manner into a small pulmonary vein without wedging. Pressures in the pulmonary artery and small pulmonary vein and changes in lung weight were continuously registered on a Sanborn Polyviso 4-channel direct writing recorder. The perfusion apparatus contained approximately one liter of blood maintained at temperatures 35-38°C by means of controlled water bath. Lungs were ventilated with a gas mixture of 95% oxygen and 5% $CO_2$ which maintained the blood pH at approximately 7.4. Injections of epinephrine and endotoxin were made intra-arterially in each study. In the second group of lung studies an intact heparinized dog was connected in series with the lung perfusion circuit. Arterial blood from the aorta was perfused at constant flow through the lung by a sigmamotor pump after appropriate cannulations in dog and lung were made. Blood from the lung was pumped to an elevated reservoir and returned *via* gravity flow to the jugular vein of the dog. Flow to the dog was maintained constant by means of a pre-set screw clamp. The mean carotid artery pressure of the dog was continuously monitored on the Sanborn recorder. An injection of endotoxin, 1.25 mg/kg body weight, was intravenously administered to the dog in each study, and the arterial blood pressure fell to hypotensive levels due to pooling effect of endotoxin(5). Experiments were terminated at approximately one hour after endotoxin. Further aspects of these experiments were identical to the pump perfused lung studies described above. Two control experiments of approximately 90 minutes each were also included to determine stability of the preparation. In the last experiments, amputated forelimbs of dogs were separately perfused with blood oxygenated by a perfused lung or pumped from a donor dog to which the venous blood was subsequently returned. Limbs were perfused at constant flow in each instance. The experimental preparation was carried out by a procedure recently described (14). Pressures in the brachial artery and small branch of the cephalic vein and changes in limb weight were continuously recorded. Limbs were maintained in the isogravimetric state prior to injection of endotoxin. Five mg of endotoxin were intra-arterially injected in the pump-lung perfused limb experiments and 1.25 mg/kg body weight were intravenously injected in the intact dog-pump-limb experiments. Freshly diluted, refrigerated synthetic epinephrine (Winthrop-Stearns, N. Y.) was used in all studies described above. Epinephrine was given in dosages sufficient to obtain accurately measurable responses. Purified *E. coli* endotoxin, prepared as previously described(5) was used in all experiments.

*Results.* Table I shows the results in 5 pump-perfused isolated lungs. Injections of

TABLE I. Vascular Responses of Pump-Perfused Dog Lung to Epinephrine before and after Injections of Endotoxin.

| | | | | Pulm. art. press. (mm Hg) | | Pulm. vein press. (mm Hg) | |
|---|---|---|---|---|---|---|---|
| Exp. | Drug | Dose (μg) | Time (min.) | Control | Max Δ | Control | Max Δ |
| 1 | epi | 5 | − 14 | 12.5 | +4.5 | 6.5 | + .5 |
| Lung wt, 312 g<br>Flow, 220 cc/min. | Endotoxin (5 mg) | | 0 | | | | |
| | epi | 5 | +14 | 17.0 | +4.0 | 7.5 | + .3 |
| | " | 5 | +27 | 16.0 | +2.5 | 8.0 | + .5 |
| 2 | epi | 5 | − 43 | 17.5 | +3.5 | 8.5 | +3.5 |
| Flow, 190 cc/min. | " | 5 | − 27 | 16.5 | +3.0 | 8.5 | +2.0 |
| | Endotoxin (5 mg) | | 0 | | | | |
| | epi | 5 | +13 | 19.0 | +3.5 | 10.0 | +3.5 |
| | " | 5 | +47 | 18.5 | +3.0 | 8.5 | +4.0 |
| 3 | epi | 4 | − 2 | 12.0 | +5.5 | 5.0 | +1.5 |
| Lung wt, 194 g<br>Flow, 180 cc/min. | Endotoxin (5 mg) | | 0 | | | | |
| | epi | 4 | +13 | 14.0 | +1.0 | 9.0 | +2.0 |
| 4 | epi | 2 | − 37 | 15.5 | +1.5 | none | |
| Lung wt, 39 g | " | 5 | − 30 | 15.5 | +2.5 | | |
| Flow, 94 cc/min. | " | 3 | − 9 | 17.0 | +2.0 | | |
| | Endotoxin (1 mg) | | 0 | | | | |
| | epi | 2.5 | + 2 | 18.0 | +1.5 | | |
| | " | " | + 8 | 16.5 | +1.5 | | |
| | " | " | +19 | 20.0 | + .5 | | |
| | " | " | +35 | 18.0 | +1.0 | | |
| 5 | epi | 4 | − 10 | 16.0 | +1.5 | none | |
| | " | 2 | − 6 | 16.0 | +1.5 | | |
| | Endotoxin (1 mg) | | 0 | | | | |
| | epi | 2 | + 8 | 17.5 | +1.5 | | |
| | " | 4 | +13 | 17.5 | +1.0 | | |

epinephrine were made before, and 2 to 47 minutes after administration of 1-5 mg of endotoxin. No significant alterations in vascular reactivity to epinephrine after endotoxin were observed, as evidenced by changes in arterial and venous pressures.

The results from 3 pump-lung experiments provided with an intact dog in the perfusion circuit are shown in Table II. Injections of epinephrine into the pulmonary artery inflow were made before and after endotoxin, administered to the dog. No marked changes in pulmonary artery response to epinephrine were observed from 5 to 65 minutes after endotoxin, although in 2 instances (Exp. 2 and 3) somewhat smaller responses were recorded than were obtained in control injections. Pulmonary venous responses were somewhat decreased in Exp. 1, and increased to a small degree in Exp. 3. However, although pre-endotoxin vascular responses to epinephrine were fairly constant in the perfused lung, spontaneous alterations in response did occur, as is readily seen in Table III. It is noted that the maximal difference in response after endotoxin (Table II) is a 50% change and that a similar change is also observed in control experiments shown in Table III, when endotoxin was not administered.

Tables IV and V illustrate results in 6 experiments on the perfused dog forelimb. In Table IV are shown 3 experiments in which injections of epinephrine were made into the perfusion tubing of a perfused lung-forelimb preparation before, and from 9 to 51 minutes after endotoxin. No changes in vascular reactivity to epinephrine were seen as evidenced by arterial and venous pressure responses.

TABLE II. Vascular Responses of the Dog-Pump-Perfused Lung to Epinephrine before and after Injections of Endotoxin.

| Exp. | Drug | Dose (µg) | Time (min.) | Mean art. B.P. of dog (mm Hg) | Pulm. art. press. (mm Hg) Control | Max △ | Pulm. vein press. (mm Hg) Control | Max △ |
|---|---|---|---|---|---|---|---|---|
| 1 | epi | 15 | −25 | 100 | 18.5 | +1.5 | 7.5 | +5.0 |
| Lung wt, 70 g | " | " | −15 | 90 | 17.5 | +1.0 | 8.0 | +4.0 |
| Flow, 110 cc/min. | " | " | − 4 | 90 | " | +1.5 | 9.0 | " |
| Dog wt, 24 kg | Endotoxin (30 mg) | | 0 | | | | | |
| | epi | " | + 8 | 85 | 18.5 | +2.0 | 8.0 | +2.5 |
| | " | " | +20 | 80 | " | +1.5 | 6.0 | +2.0 |
| | " | " | +28 | 70 | 19.0 | +1.0 | 6.5 | +1.5 |
| | " | " | +36 | 70 | " | +1.5 | 7.5 | " |
| | " | " | +54 | 67 | 19.5 | " | 7.0 | " |
| 2 | epi | 40 | −24 | 90 | 20.5 | +3.0 | 8.5 | +3.0 |
| Lung wt, 42 g | " | " | − 8 | 125 | 22.5 | +2.0 | 10.0 | " |
| Flow, 90 cc/min. | Endotoxin (20 mg) | | 0 | | | | | |
| Dog wt, 16 kg | | | | | | | | |
| | epi | " | + 9 | 50 | 22.5 | +3.5 | 12.0 | +2.0 |
| | " | " | +22 | 65 | 21.5 | +1.0 | 11.0 | " |
| | " | " | +35 | 90 | 22.5 | +1.5 | 8.5 | +2.5 |
| | " | " | +64 | 60 | 20.5 | +3.0 | 9.5 | +3.0 |
| 3 | epi | 20 | −27 | 165 | 11.0 | +4.0 | 5.0 | +1.5 |
| Lung wt, 125 g | " | " | −13 | 170 | 12.0 | +4.5 | 6.0 | " |
| Flow, 120 cc/min. | " | " | − 3 | 160 | 12.5 | +4.0 | 6.0 | +2.0 |
| Dog wt, 24 kg | Endotoxin (30 mg) | | 0 | | | | | |
| | epi | " | + 5 | 35 | 15.0 | +2.5 | | |
| | " | " | +12 | 95 | 16.5 | +3.0 | 12.5 | +1.0 |
| | " | " | +28 | 120 | 13.5 | " | 8.0 | +3.0 |
| | " | " | +45 | 90 | 12.0 | +4.0 | 7.0 | +4.0 |
| | " | " | +58 | 70 | 11.5 | +3.5 | 6.0 | " |
| | " | " | +65 | 45 | 12.5 | +2.5 | 7.0 | +3.0 |

Table V shows 3 experiments on the isolated perfused forelimb with an intact dog providing arterial blood for the limb. No significant differences in vascular response to epinephrine were observed from 16 to 60 minutes after endotoxin, except that in one experiment (#1) the limb artery response to epinephrine decreased by about 50%. Dogs

TABLE III. Vascular Responses of Dog-Pump-Perfused Lung to Epinephrine in Absence of Endotoxin (Controls).

| Exp. | Drug | Dose (µg) | Time (min.) | Mean art. B.P. of dog (mm Hg) | Pulm. art. press. (mm Hg) Control | Max △ | Pulm. vein press. (mm Hg) Control | Max △ |
|---|---|---|---|---|---|---|---|---|
| 1 | epi | 20 | 0 | 115 | 16.5 | + 3.5 | 6.0 | +7.5 |
| Lung wt, 41 g | " | " | + 6 | 120 | 17.0 | + 3.0 | 5.5 | " |
| Flow, 85 cc/min. | " | " | +15 | 117 | " | " | 6.5 | +9.0 |
| Dog wt, 26 kg | " | " | +30 | 140 | 16.0 | " | 4.0 | +7.0 |
| | " | " | +45 | 145 | 15.0 | + 3.5 | 3.5 | " |
| | " | " | +58 | 128 | " | " | 4.0 | " |
| | " | " | +71 | 130 | " | + 4.5 | " | " |
| | " | " | +83 | 135 | " | " | " | " |
| 2 | epi | 20 | 0 | 130 | 18.0 | + 6.5 | 10.0 | +3.0 |
| Lung wt, 75 g | " | " | +11 | 133 | 16.0 | + 9.5 | " | " |
| Flow, 175 cc/min. | " | " | +25 | 125 | 15.0 | +10.0 | 8.0 | +4.0 |
| Dog wt, 17 kg | " | " | +44 | 130 | 14.0 | + 9.5 | 9.0 | +2.0 |
| | " | " | +55 | 127 | 13.5 | + 9.0 | 8.5 | +3.5 |
| | " | " | +71 | 110 | 13.0 | " | 7.0 | +4.0 |
| | " | " | +80 | 115 | 14.0 | + 7.5 | 8.5 | " |
| | " | " | +92 | 133 | 13.5 | + 8.5 | 6.0 | +5.0 |

TABLE IV. Vascular Responses of Pump-Lung-Perfused Dog Forelimb to Epinephrine before and after Injections of Endotoxin.

| | | | | Brach. art. press. (mm Hg) | | Cephalic vein press. (mm Hg) | |
|---|---|---|---|---|---|---|---|
| Exp. | Drug | Dose (μg) | Time (min.) | Control | Max △ | Control | Max △ |
| 1 | epi | 1 | − 35 | 55 | +18 | 14.0 | −3.5; +2.0 |
| Limb wt, 397 g<br>Flow, 30 cc/min. | Endotoxin (5 mg) | | 0 | | | | |
| | epi | 1 | +32 | 53 | +19 | 15.0 | −2.0; +5.5 |
| 2 | epi | 1 | − 17 | 55 | +25 | 9.0 | −1.0; +6.0 |
| Flow, 53 cc/min. | Endotoxin (5 mg) | | 0 | | | | |
| | epi | 1 | + 9 | ” | +31 | ” | −2.0; +2.0 |
| | ” | 1 | +36 | ” | +33 | 10.0 | ” ; ” |
| | ” | 1 | +51 | 57 | +32 | 9.5 | −2.5; +5.5 |
| 3 | epi | .1 | − 37 | 90 | +23 | 5.0 | +.5 |
| Flow, 19 cc/min. | ” | .1 | − 14 | 113 | +46 | 3.0 | ” |
| | Endotoxin (5 mg) | | 0 | | | | |
| | epi | .1 | +12 | 107 | +30 | 4.0 | ” |

were markedly hypotensive following injection of endotoxin.

*Discussion.* These experiments fail to show a significant degree of altered vascular reactivity to epinephrine following injection of endotoxin in the isolated perfused dog forelimb and lung. The largest differences in response were of a 2:1 magnitude, however,

TABLE V. Vascular Responses of the Dog-Pump-Perfused Forelimb to Epinephrine before and after Injections of Endotoxin.

| | | | | | Brach. art. press. (mm Hg) | | Cephalic vein press. (mm Hg) | |
|---|---|---|---|---|---|---|---|---|
| Exp. | Drug | Dose (μg) | Time (min.) | Mean art. B. P. of dog (mm Hg) | Control | Max △ | Control | Max △ |
| 1 | epi | .25 | − 32 | 137 | 36 | + 47 | 11.5 | + 9.0 |
| Limb wt, 595 g | ” | ” | − 24 | 125 | 39 | + 34 | | |
| Flow, 44 cc/min. | ” | .50 | − 15 | 132 | 50 | + 75 | 18.0 | +13.0 |
| Dog wt, 30 kg | ” | ” | − 8 | 127 | 44 | + 60 | 20.0 | + 5.5 |
| | Endotoxin (40 mg) | | 0 | | | | | |
| | epi | ” | +17 | 65 | 100 | + 30 | 29.0 | +11.0 |
| | ” | ” | +39 | 58 | 84 | + 26 | 30.5 | + 8.5 |
| | ” | ” | +55 | 107 | 48 | + 32 | 33.0 | + 9.0 |
| | ” | ” | +60 | 110 | 45 | + 35 | 31.5 | +11.0 |
| | ” | 1.0 | +65 | 100 | 48 | + 56 | ” | +16.5 |
| 2 | epi | .5 | − 16 | 142 | 72 | +118 | 10.0 | + 9.5 |
| Limb wt, 392 g | ” | ” | − 9 | 150 | ” | +128 | 10.5 | +11.5 |
| Flow, 39 cc/min.<br>Dog wt, 19.2 kg | Endotoxin (24 mg) | | 0 | | | | | |
| | epi | ” | +18 | 65 | 155 | + 85 | 14.5 | + 8.5 |
| | ” | ” | +33 | 103 | 127 | + 76 | ” | + 8.0 |
| | ” | ” | +44 | 102 | 80 | +106 | 11.0 | + 9.0 |
| | ” | ” | +60 | 142 | 75 | +108 | 14.0 | +11.0 |
| 3 | epi | 1.0 | − 33 | 115 | 110 | + 52 | 23.0 | +12.5 |
| Flow, 20 cc/min. | ” | ” | − 27 | 100 | 84 | + 44 | | |
| Dog wt, 28 kg | ” | ” | − 21 | 90 | 104 | + 66 | 16.5 | +17.0 |
| | ” | ” | − 10 | 98 | 114 | + 51 | 19.0 | +10.5 |
| | Endotoxin (35 mg) | | 0 | | | | | |
| | epi | ” | +16 | 50 | 108 | + 50 | 20.5 | +13.5 |
| | ” | ” | +22 | 10 | 82 | + 40 | 20.0 | +11.0 |

these do not appear significant in view of similar spontaneous changes. Experiments were not extended beyond one hour after endotoxin and do not therefore rule out possible later changes in vascular response to epinephrine. It is of interest to note however, that since a significant fall in peripheral resistance occurs within 30 minutes after endotoxin in the eviscerated dog(13), data of this study fail to suggest a relationship between progressive decline in total peripheral resistance and an altered reactivity of blood vessels to circulating epinephrine.

It should be pointed out that rather large amounts of endotoxin were used. However, large changes in vascular reactivity have been reported in other studies in which the endotoxin dosage was large enough to produce death(1). Zweifach, Nagler, and Thomas reported 100-fold changes in vascular reactivity with 2 mg injections of endotoxin in the rat mesentery(1), and even larger masses of tissue and greater volumes of blood were involved in the dog limb and lung studies than in the rat experiments.

Additional experiments were performed in which combinations of epinephrine, nor-epinephine and 5-hydroxytryptamine were injected before and after endotoxin, but no potentiation or depression of response to these simultaneously injected drugs occurred in the doses and ratios of doses employed. Single intra-arterial injections of nor-epinephrine and 5-hydroxytryptamine were also separately made in isolated perfused lungs, limbs and kidneys of dogs before and after injections of endotoxin. No changes in vascular response were observed up to one hour after endotoxin.

A complicating factor in the present study is that endotoxin may increase vessel tone (Exp. 3, Table II) so that pressure responses following subsequent injection of epinephrine, may not be directly comparable to those obtained before administration of endotoxin. Nonetheless, since organs were perfused at constant flow, changes in perfusion pressure after epinephrine represent changes in resistance. These changes can be compared with each other although they do not necessarily represent comparable changes in the tone of the vascular smooth muscle.

It may be that the differences between findings of the present study and those of Zweifach and others are due either to the kind of experimental preparation used or to the species employed. In any event it appears that alterations in vascular reactivity to epinephrine may not be observed in all species and are probably unimportant in the dog.

*Summary.* Experiments were carried out on isolated perfused lungs and forelimbs of adult dogs. There was no significant change in vascular response to epinephrine following injection of endotoxin.

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# Effect of Dietary Composition on Rat Liver Phosphorylase Activity.
(24464)

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In continuation of our studies on changes in enzymatic activity due to dietary adaptation we investigated the effect of feeding fat and carbohydrate diets on activity of liver phosphorylase. One of us recently reported that during absorption of fat in the rat the activity of liver alkaline phosphatase increased as compared to that of carbohydrate fed animals(1). This activity returned to normal after 12 hour fast, even though the animals had been adapted to the fat or carbohydrate diets over an extended period. Since these results failed to indicate the presence of a specific adaptative mechanism, we could only attribute the increase in enzyme activity to a transitory effect of fat absorption. It has also been shown that substitution of protein, fat, galactose or fructose in the diet for a direct glucose source, produces an increase in activity of liver glucose-6-phosphatase in the rat(2). Adaptation to a high fat diet increases rate of utilization of fat and decreases that of carbohydrate in normal and hypophysectomized rats during subsequent fasting period(3). Fat adapted rats are also able to maintain more efficiently their blood sugar levels during periods of glycogenolysis (4). A correlation between level of phosphorylase activity and rate of glycogen breakdown has been found by several investigators. When liver slices are incubated in a medium containing high levels of Na there is an increase in phosphorylase activity parallel with increase in rate of glycogenolysis(5). Increases in rate of glycogenolysis and in phosphorylase activity were also shown to take place in liver of obese hyperglycemic mice (6). Pancreas HGF, epinephrine and other sympathomimetic drugs induce similar effects (7). This apparent relationship between activity of liver phosphorylase and rate of glycogenolysis suggested the possibility of associating this enzyme with metabolic and biochemical changes that occur as a consequence of adaptation of rats to fat or carbohydrate diets.

*Materials and methods.* Animals used were albino rats of CNEA strain. They were housed in individual cages. High fat and high carbohydrate diets were similar to those of previous experiments†(1). In long term feeding experiments, diets were offered *ad lib.* for 45 days. Growth was satisfactory for both groups. For determination of phosphorylase activity, rats were sacrificed by a blow on the neck. The liver was removed, homogenized in a Potter-Elvehjem homogenizer at high speed, and diluted with 0.1 M NaF to obtain a 33% homogenate. During homogenization the tube was immersed in ice bath. The phosphorylase activity was determined not later than 1 hour after removal of liver. For determining enzymatic activity an aliquot of the homogenate (0.1-0.2 ml) was incubated with a substrate containing 0.022 M glucose-1-phosphate,‡ 0.073 M citrate buffer (pH 6.2) and 0.083 M NaF, in total volume of 0.65 ml, for 30 minutes at 38°C. Fluoride was used for its ability to inhibit phosphoglucomutase(8) and acid phosphatase (9). At end of incubation, enzyme activity was stopped by addition of 1 ml of 10% TCA. Inorganic phosphate was determined in supernatant by the method of Fiske and Subba-Row(10). A phosphorylase activity unit or micromolar unit is the amount of enzyme that will liberate 1 $\mu$M of P from Gl-1-P in the presence of fluoride, during incubation of 30 minutes at 38°C at pH 6.2.

*Results.* Table I shows effects of feeding 3 g of either fat or carbohydrate diet to rats previously fasted for 24 hours. These results are compared to those obtained with unfed fasted rats that had been previously maintained on

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‡ Gl-1-P was a generous gift of Sigma Chem. Co., St. Louis, Mo.

TABLE I. Effect of a Single Meal of a High Fat or a High Carbohydrate Diet, and of Fasting on Liver Phosphorylase Activity during Absorption.

| Group | No. rats | Diet | Units of phosphorylase/g liver |
|---|---|---|---|
| I | 8 | High CHO | 167 (±46 ) |
| II | 11 | " fat | 16.3 (± 7.7) |
| III | 20 | | 21.6 (± 7.8) |

regular stock diet. The animals fed the test meal were sacrificed 3 hours after feeding. In all instances food was still present in stomach and intestines at this time. A significant increase in phosphorylase activity was established in the carbohydrate fed group as compared to that of fat fed and non-fed animals.

Table II summarizes effects of dietary adaptation and a subsequent 24-hour fast on activity of liver phosphorylase. These results show that fat adapted animals maintain a higher activity than carbohydrate fed rats. In this case adaptation has seemed to change radically the pattern of enzyme activity, as seen by comparing these results with those obtained with the non-adapted animals in Table I.

*Discussion.* Carbohydrate feeding greatly stimulated activity of liver phosphorylase during the absorptive period, while feeding of fat seemed to slightly depress such activity. This increase of phosphorylase activity which follows ingestion of carbohydrate is compatible with augmentation of glucose metabolism that presumably occurs in liver as a consequence of increased supply of glucose.

When animals were adapted to fat or carbohydrate diets the level of enzyme activity during fasting was significantly higher in animals fed high fat diet. This increase in phosphorylase activity would seem to result from adaptive adjustment which takes place in the animal to conform its metabolic pattern to its specific dietary intake, and could conceivably explain the mechanism by which the fat adapted rat is able to maintain its glycogen stores and blood sugar at higher levels during fasting than the carbohydrate adapted animal.

TABLE II. Effect of Prolonged Feeding of High Fat and High Carbohydrate Diets on Liver Phosphorylase Activity after 24 Hours Fasting.

| Group | No. rats | Diet | Adaptation period, days | Units of phosphorylase per g liver |
|---|---|---|---|---|
| I | 7 | High fat | 45 | 133 (±55 ) |
| II | 5 | " CHO | " | 24.6 (± 6.7) |

*Summary.* During absorption of carbohydrate by the rat there is a significant increase of liver phosphorylase activity. When animals were adapted to high fat and high carbohydrate diets over 45 days and were then fasted 24 hours, the fat adapted rats maintained a higher level of liver phosphorylase activity. These changes in enzymatic activity would seem to be consistent with metabolic and biochemical changes which have been shown to occur as a consequence of the specific dietary composition.

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# Genetic Differences in Hemoglobin as Markers for Bone Marrow Transplantation in Mice. (24465)

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Immunological, histochemical, and cytological methods have been used to demonstrate the transplantation and repopulation of erythrocytic(1-4), platelet(5), thymocytic (6), and white blood cell(7,8) precursors from bone marrow cells of homologous or heterologous origin. The immunohematological systems now used to type mouse erythrocytes are based on differences at the *H-2* locus(9,10). This system permits distinction of cells of donor origin from those of the recipient when donor and recipient are of distinguishable *H-2* genotypes. Although chromosomal markers(7) may be used, in cases in which donor and recipient are indistinguishable at the *H-2* locus, the technic cannot be applied to the circulating red cells. An independent method for distinguishing erythrocytes is, therefore, potentially useful in study of bone marrow transplantation, particularly where the *H-2* markers cannot be used. With paper electrophoretic technics, Gluecksohn-Waelsch *et al.* (11) identified inheritable hemoglobin differences in mice. Starch gel electrophoresis, described by Smithies(12), can also be used for identifying hemoglobin differences in mice (Fig. 1). This preliminary report indicates that erythrocytes of mouse chimeras can be typed by using inheritable differences occurring in mouse hemoglobin.

*Methods.* Mice used as donors and recipients in this experiment were strains C57BL/S, 101, and (C57BL/S × 101)$F_1$. Erythrocytes of C57BL/S mice are of *H-2*$^b$ genotype and have the "single" type of hemoglobin, whereas erythrocytes of 101 mice seem to be of *H-2*$^k$ genotype and have the "diffuse" type. Erythrocytes of (C57BL/S × 101) $F_1$ also have diffuse hemoglobin. Donor bone marrow or splenic suspensions were injected intravenously and intraperitoneally, respectively, into X-irradiated recipients. Blood samples for serological typing and starch gel electrophoresis were obtained by severing the tail vein.

*Results.* Table I gives comparative results obtained in the 20 mice cross-examined by serological technics(3) and by electrophoresis. The data show that, when bone marrow or splenic suspensions from a donor with one type of hemoglobin are transplanted into irradiated mice that differ at the *H-2* locus and have another type of hemoglobin, the hemoglobin in the recipients' circulating erythrocytes, characterized by starch gel electrophoresis, corresponds with the type of the red blood cells as characterized serologically. The electrophoretic method has not yet been refined sufficiently to detect small quantities (20% or less) of the single type hemoglobin when 80% or more of the red blood cells have the diffuse type. However, both hemoglobin type and red cell serotype seem to be under the autonomous control of the genotype of the nucleated cell from which the erythrocyte was derived. Makinodan and Anderson(13) also found that hemoglobin in mouse-grown

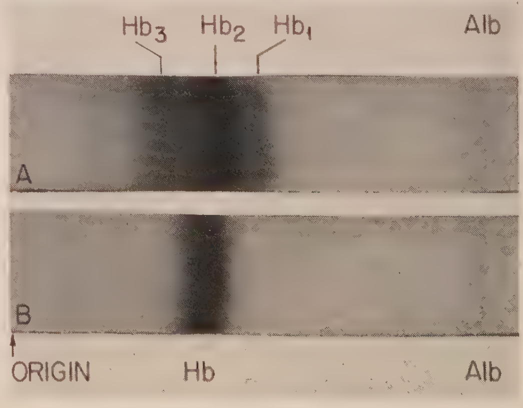

FIG. 1. Comparative patterns of mouse hemoglobins after electrophoresis in starch gel. Strip A shows the "diffuse" type of hemoglobin of strain 101 mice that resolves into 3 electrophoretically separable fractions. Strip B illustrates the "single" type of hemoglobin of C57BL/S mice (R. Popp and W. St. Amand, unpublished data).

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* Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

TABLE I. Comparison of Serotype and Hemoglobin Type in X-irradiated Mouse Chimeras.

| Recipient mice | | | Donor cells (× $10^6$ nucleated cells) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Bone marrow | | Spleen | | Post | Serotype | | Hemoglobin type | |
| No. | Strain | Dose (r) | C57BL/S | (C57BL/S × 101)$F_1$ | C57BL/S | (C57BL/S × 101)$F_1$ | treatment (days) | Recipient (%) | Donor (%) | Recipient | Donor |
| 2 | 101 | 850 | 15 | | | | 43 | 100 | | × | |
| 2 | (C57BL/S × 101)$F_1$ | 900 | 15 | | | | 93 | " | | × | |
| 2 | | " | 15 | | | | " | | 100 | | × |
| 1 | | " | 15 | | | | 142 | 3 | 97 | | × |
| 1 | C57BL/S | " | | 15 | | | 42 | | 100 | | × |
| 1 | (C57BL/S × 101)$F_1$ | 400 | | | 30 | | 93 | 100 | | × | |
| 1 | | " | | | " | | " | 93 | 7 | × | |
| 1 | | " | | | " | | " | | 100 | | × |
| 1 | | 500 | | | " | | 21 | 100 | | × | |
| 1 | | " | | | " | | " | 64 | 36 | × | × |
| 1 | | " | | | " | | " | 62 | 38 | × | × |
| 1 | | " | | | " | | " | 80 | 20 | × | |
| 1 | | 600 | | | " | | 93 | 100 | | × | |
| 3 | C57BL/S | 400 | | | | 120 | 35 | " | | × | |
| 1 | | " | | | | " | " | 97 | 3 | × | |

rat erythrocytes has physicochemical properties of rat hemoglobin. Origin (host or recipient) of the circulating erythrocytes can therefore be inferred from the hemoglobin type as well as from the serotype. Thus, after transplantation of bone marrow cells between strains of mice with identical *H-2* genotypes, one can infer whether the circulating erythrocytes are of donor or recipient origin if there are differences in their hemoglobin types; *e.g.*, C57BL/6 and 129/Rr are both *H-2*$^b$, the former has a single and the latter a diffuse hemoglobin.

Snell *et al.*(14) and Counce *et al.*(15) showed that the *H-2* locus strongly affects transplantability of tumors and skin in mice. Trentin(16) and Uphoff(17,18) suggested that the *H-2* antigens may also be important in reactions occurring after bone marrow transplantation into lethally irradiated mice. Bone marrow transplantations between sublines of mice indistinguishable at the *H-2* locus but with different hemoglobin types and varying degrees of heterogeneity at other loci may further help to elucidate the relative importance of the *H-2* locus and other histocompatibility loci in bone marrow transplantation.

*Summary.* Both hemoglobin type and red cell serotype seem to be autonomously controlled by the genotype of the nucleated cell from which the erythrocyte is derived. Thus, genetic differences in hemoglobin can be used as markers for bone marrow transplantation in irradiated mice. Hemoglobin typing may be particularly useful where the *H-2* markers cannot be used.

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## Incorporation of Cystine and Lysine by Normal and "Cystinuric" Leukocytes. (24466)

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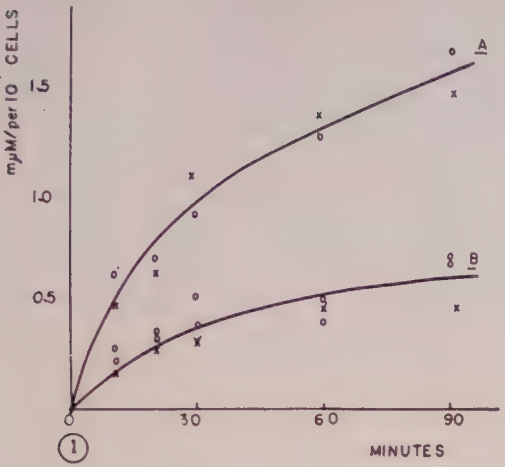
Cystinuria has been shown by Dent(1,2) to be a disease of renal tubules in which amino acids cystine, lysine, arginine and ornithine fail to be absorbed from the glomerular filtrate. Since these substances can be considered as structurally related di-amino acids(3) and their absorption from the glomerular filtrate is largely controlled by a single gene(4), it has been suggested that a single mechanism for absorption of these amino acids exists in the renal tubules. However, other kinds of body cells are capable of concentrating amino acids, such as muscle(5), white blood cells (6), tumor cells(7), and established cell lines grown *in vitro*(8). If the concentration mechanism of amino acids by such cells were related to that of the kidney tubules, these cells might also be affected in the cystinuric individual, for biochemical defects in genetic diseases are frequently widespread. The fact that such a general metabolic abnormality has not been found in cystinuric subjects does not argue against this possibility, for whereas the defect in kidney tubules results in obvious failure to transport amino acids against a concentraton gradient into the blood, the other body cells might continue to obtain sufficient amino acids from blood by diffusion alone, the blood level of these amino acids in cystinurics remaining normal or only slightly reduced(2,9). If however, such cells were placed in a medium containing a considerably lower amino acid concentration than normal blood, any latent defect of this kind in the cystinuric cells might become evident. In the present experiment, therefore, white blood cells of normal and cystinuric subjects were compared in their abilities to incorporate lysine and cystine from the medium, at concentrations below those existing normally in human blood, and at which rate of uptake depends upon concentration in the medium. At the lowest concentration tested, uptake of amino acids by normal and "cystinuric" leukocytes was the same.

*Methods.* The cystinuric subjects were a brother and sister in their forties who had an extensive family history of the disease; each had been operated on more than once for renal stones, but otherwise was in good health. Their urine gave a strong cyanide-nitroprusside test for cystine. White blood cells were separated from freshly drawn human blood by fibrinogen sedimentation as described by Skoog and Beck(10). They were resuspended in isotonic sodium chloride solution and washed twice. The final suspension was made in the balanced salt solution of Eagle, BSS,(11) and the cells incubated with gentle shaking in water bath at 37°C, under atmosphere of 5% $CO_2$, 95% $O_2$. At time zero, labeled amino



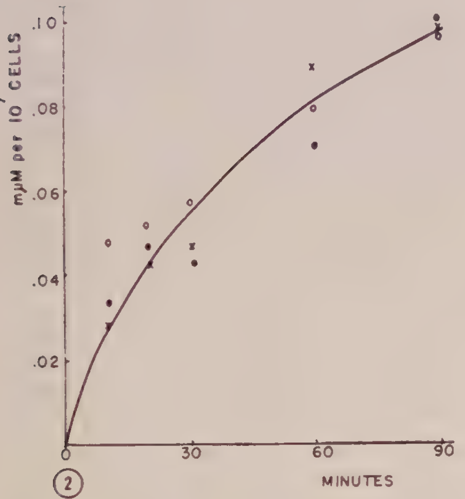


FIG. 1. Lysine incorporation into normal and cystinuric leukocytes. Medium concentration of 2-$C^{14}$ DL-lysine (0.6 mC/mM). Curve A—2.2 × $10^{-4}$ molar. Curve B—0.43 × $10^{-4}$ molar. ○—normal leukocytes. ×—cystinuric leukocytes. Ordinate represents mμmoles of amino acid taken up by the cells.

FIG. 2. Cystine incorporation into normal and cystinuric leukocytes. Medium concentration of $S^{35}$ L-cystine (3.84 mC/mM) 7.1 × $10^{-6}$ molar. ●—normal leukocytes in absence of lysine and arginine; ○—normal leukocytes and ×—cystinuric leukocytes, each in presence of 2.5 × $10^{-3}$ molar L-lysine and L-arginine.

acid was added, and at intervals aliquots were diluted into 20 ml cold BSS and plated and washed on a membrane filter (millipore). The infinitely thin samples were air dried, mounted on planchets, heated in forced draft oven at 70°C, one hour, and then counted. (Previous experiments had shown that substantially all the counts at these concentrations were insoluble in hot 5% trichloroacetic acid, and therefore had become incorporated into cell protein.) From counts/minute in the sample, and the specific activity of amino acid added, the molar quantities taken up were calculated.

*Results.* Fig. 1 shows uptake of lysine at 2 different concentrations in the medium.

Incorporation began immediately at maximum rate but slowed considerably by 90 minutes. At these low concentrations of lysine, rate of uptake depended on concentration, as previously shown for leucine uptake by white blood cells(12). Rate of uptake at a DL-lysine concentration of 0.43 x $10^{-4}$M was only about half that at 2.2 x $10^{-4}$M, but at both concentrations the cystinuric white blood cells took up lysine as well as normal ones.

Uptake of cystine was followed at concentrations as low as 7.1 x $10^{-6}$M and the white blood cells of cystinuric subjects took up cystine at a normal rate. Lysine and arginine added to the medium in high concentrations (2.5 x $10^{-3}$M) did not affect rate of uptake of cystine by either normal or cystinuric white cells (Fig. 2).

It appeared, therefore, that no defect could be demonstrated in uptake of lysine and cystine by cystinuric white blood cells, at concentrations considerably below normal blood concentration(9). In addition, there was no demonstrable effect of arginine or lysine on cystine uptake by either cystinuric or normal white blood cells, although such competition occurs in the renal tubules(13). If, as seems likely, cellular concentrating mechanisms for amino acids play a role in determining rate of entry of amino acids into cells(14) then these mechanisms may be basically different from those involved in tubular absorption of amino acids from the glomerular filtrate, or, if they are similar, a defect in such a mechanism might exist in tubular cells alone.*

*Summary.* Leukocytes of cystinuric patients incorporate labelled cystine and lysine from the medium at a normal rate, even when these amino acids are added in very low concentration. Rate of uptake of cystine by either normal or "cystinuric" leukocytes is not affected by presence of high concentrations of arginine and lysine. The mechanism of renal tubular concentration may therefore be different in kind from that of leukocytes.

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* It is interesting to note that in a recent study of the concentrating power of human cells cultivated *in vitro* (HeLa) the amino acids involved in cystinuria formed a distinct group as the most poorly concentrated of all the amino acids(8).

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## Effects of Handling on the Adrenalectomized Rat.* (24467)

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Previous studies using normal young albino rats have shown that handling or gentling results in significant increases in weight gain and skeletal length(1,2), superior learning ability(3), and increased resistance to certain forms of stress(4,5). It has recently been shown that hypophysectomy alters some of the response to handling(6). The purpose of this paper is to report the influence of adrenalectomy on certain effects induced by handling.

*Methods.* Three consecutive experiments were done using young male rats of Sprague-Dawley strain.† Bilateral adrenalectomy was performed in a single operation on 23rd day of life in Exp. I and on 21st day in Exp. II and III. The animals were weighed and then divided into 2 equal groups: 1) Beginning on day following adrenalectomy each animal of experimental group was handled individually for 10 minutes every day at same time of day for 2 months. In each experiment a different investigator handled the animals. Handling consisted of holding the animal in one hand and stroking it repeatedly with the other hand from head to tail with animal facing the handler. 2) Control animals were never touched after their original placement in cages following adrenalectomy. A weighed amount of food in dish (Exp. I and II) or special feeding cup‡ to minimize spillage (Exp. III) was placed in each cage and leftover food weighed to determine individual daily food consumption. Food used in Exp. No. I and II was as follows: 480 g canned dog food (Pard®), 2 slices of crumbled whole wheat bread, and 2.5 ml Vi-Daylin® (liquid multivitamin preparation marketed by Abbott Labs.). These ingredients were thoroughly mixed and formed a soft, adherent mass such that food loss from spillage was minimal. In Exp. III powdered Purina Rat Chow was used. In all experiments 5% glucose in normal saline was supplied as drinking water. Animals were housed

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† Obtained from Hormone Assay Labs., Chicago.

‡ Obtained from A. S. Aloe and Co.

TABLE I. Effect of Handling on Adrenalectomized Rats.

| Exp. No. | I | | | II | | | III | | |
|---|---|---|---|---|---|---|---|---|---|
| Diet | Pard | | | Pard | | | Purina | | |
| Exp. group | Not handled | Handled | p† | Not handled | Handled | p† | Not handled | Handled | p† |
| No. of rats—Initial No. | 14 | 13 | | 11 | 11 | | 14 | 14 | |
| Final " | 14 | 13 | | 8 | 9 | | 6 | 8 | |
| Initial wt (g)* | 49.2 ± 7.1 | 47.9 ± 6.9 | .7 | 47.0 ± 6.1 | 48.5 ± 7.5 | .6 | 45.8 ± 2.6 | 45.7 ± 3.2 | .9 |
| Wt gain (g)* | 164.5 ± 35.4 | 166.0 ± 25.4 | .9 | 135.0 ± 40.7 | 140.0 ± 51.5 | .9 | 187.0 ± 42.7 | 174.0 ± 45.9 | .6 |
| Total food consumed (g)* | 2173.0 ± 179.5 | 1999.0 ± 277.0 | .1 | 1453.0 ± 162.0 | 1512.0 ± 182.0 | .5 | 565.0 ± 59.0 | 521.0 ± 110.0 | .4 |
| Food consumed (g)/g wt gained* | 13.7 ± 3.2 | 12.2 ± 1.9 | .2 | 11.3 ± 2.6 | 11.6 ± 2.8 | .9 | 3.0 ± .3 | 3.0 ± .3 | .9 |
| Maze learning—No. animals tested | 14 | 13 | | | | | 5 | 6 | |
| Trials to criterion* | 103.0 ± 30.0 | 120.0 ± 38.0 | .2 | | | | 130.0 ± 30.0 | 118.0 ± 24.8 | .5 |
| No. of errors in first 100 trials* | 30.0 ± 7.3 | 33.0 ± 10.3 | .4 | | | | 33.0 ± 9.9 | 28.0 ± 5.6 | .4 |
| Thiourea toxicity—No. animals tested | 14 | 13 | | | | | 5 | 6 | |
| Mortality | 5 | 5 | .9 | | | | 1 | 2 | .9 |

* Mean ± stand. error.

† Significance of difference between handled and unhandled groups.

individually in wire cages arranged in rack so that handled and unhandled animals alternated. The animals were weighed weekly in their cages and weight of cages subtracted to avoid handling. The temperature of animal room was maintained at 80 ± 2° F. After 6 weeks animals of each group were tested on a black-white discrimination problem. The apparatus was a T-shaped discrimination box, described elsewhere(3). Either arm of the T could be lighted, and the training was light-positive. The sequence of turns for the lighted alley was L (left) R (right) RLLRLRRL for odd tens of trials and RLLRRLRLLR for even tens. Animals were allowed 5 runs a day for the first 2 days and 10 runs a day thereafter, until each animal reached the criterion of learning (2 successive errorless days of 10 trials each), except that all animals were given at least 100 trials. Record was also kept of number of errors made by each animal. An error was considered to have been made if the animal's body, exclusive of tail, entered the unlighted T-arm. Following completion of testing, animals in Exp. I and III were tested for resistance to a single intraperitoneal dose (50 mg/kg) of thiourea as previously described(7). At end of experiment, the animals were sacrificed and examined for residual adrenal tissue.

*Results.* Table I shows the data from the 3 experiments. There was no significant difference in food consumption, weight gain, maze learning ability, or resistance to thiourea toxicity between handled and unhandled animals.

Small amounts of residual or regenerating adrenocortical (but no medullary) tissue were found at autopsy in animals from each experiment. Since the autopsies were done about one month after experiments were completed, it is assumed that all animals were deficient in adrenal hormones during the study. A comparison of animals with and without residual adrenal tissue with respect to weight gain, food consumption, maze learning ability and resistance to thiourea toxicity revealed no significant difference.

The results of our experiments indicate that a certain minimum level of adrenal hormones is necessary for the enhancing action of handling on weight gain, efficiency of food utilization, maze learning ability and resistance to thiourea toxicity previously reported in the normal young albino rat. Whether the effects of handling in question depend on presence of adrenal medullary and/or adrenocortical hormones remains to be determined. The fact that significant effects of handling on weight gain and food utilization occur in the hypophysectomized rat(6) does not resolve this problem. It could be argued that because these changes occur in the absence of ACTH, adrenal medullary hormones (not known to be influenced by hypophysectomy) are the crucial factors. On the other hand, it could be postulated that the diminished effects observed in the hypophysectomized rat (as compared to normal rat) implicate the adrenocortical hormones known to be diminished by hypophysectomy.

*Summary.* Experiments indicate that the adrenal gland is necessary for the enhancing effects on weight gain, efficiency of food utilization, maze learning ability and resistance to thiourea toxicity previously shown to result from handling, in the intact young albino rat.

Addendum: Experiments completed since this manuscript was submitted indicate that handling does not regularly cause an increased weight gain in normal rats (Sprague-Dawley and Wistar strains) whereas it does consistently increase learning ability and resistance to stress. These findings indicate that the lack of effect of handling on weight gain in the experiments reported in this paper may be due to factors other than the absence of the adrenal glands.

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# Susceptibility of Garter Snakes (*Thamnophis* Spp.) to Western Equine Encephalomyelitis Virus. (24468)

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(Introduced by C. L. Larson)
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In temperate zones, the importance of a mosquito-bird-mosquito cycle for disseminating western equine encephalomyelitis (WEE) virus in summer is well established(1). A major problem, however, in a study of the ecology of WEE virus is the overwintering mechanism. One such mechanism suggested has been a continuing bird-mosquito-bird cycle in warmer climates with seasonal invasion of colder climates through migrating birds. Reeves *et al.*(2) reported that chronic latent infection of 1 to 10 months' duration occasionally occurs in wild birds experimentally infected with WEE virus and suggested that this may account for maintenance of virus during winter months. However, Kissling *et al.*(1) did not isolate WEE virus from blood of 764 birds wintering in southern U. S. or from 739 birds entering the same area on their spring migration. The relative paucity of evidence that birds served as a winter reservoir of virus or reintroduced it into northern areas each year suggested a consideration of hibernating animals. With regard to mosquitoes themselves, evidence is mounting that they are relatively unimportant as a winter reservoir of infection(3,4,5,6). Hibernating vertebrates were considered, since on theory of latent infection(2), such species, if susceptible, might serve to maintain virus during winter months and to disperse it when vectors become active. Field studies showed that *Culex tarsalis,* the mosquito vector, overwinters in rock piles(5) and that many snakes

TABLE I. Susceptibility of Garter Snakes (*Thamnophis* spp.) to Western Equine Encephalomyelitis Virus.

| Snake No. | Method of infection | Dosage | Days after feeding or inoc. on which blood tested for presence of virus† | Highest dilution in which virus was detected* | (day) | Remarks |
|---|---|---|---|---|---|---|
| 2 | Bites, 9 mosquitoes | | (1),4,5,6,8,12,13,15, 18,20,21,22,23 | $10^{-2}$ | 21 | Died 23rd day |
| 94 | " 2 " | | 3,6,8,13,16 | $10^{-6}$‡ | 13 | " 16th " |
| 27 | " 20 " | | 4,7 | $10^{-3}$‡ | 7 | " 8th " |
| 1 | I.P. inoculation | $10^6$LD$_{50}$ | 3,7,(13,14,15,17,21) | N.D. | | Survived |
| 40 | *Idem* | " | 4,6,12 | $10^{-7}$‡ | 12 | Died 15th day |
| 42 | " | " | 4,6,12,15,(18,22) | $10^{-4}$ | 12 | Survived |
| 50 | " | $10^4$LD$_{50}$ | 10,36 | N.D. | | Died 36th day |

* Generally only one blood specimen was checked for amount of virus present.
† Days on which no virus was found are enclosed in parentheses.
‡ Highest dilution tested.
N.D. = Not determined.

also hibernate in these sites. Laboratory investigations revealed that *C. tarsalis* feeds on garter snakes in absence of other hosts(7) and Dow *et al.*(8) collected blood-engorged *C. tarsalis* in snake-baited traps. This preliminary report presents data showing that garter snakes can be infected readily either by bites of infected *C. tarsalis* or by intraperitoneal inoculation of a recently isolated mosquito strain of WEE virus.

*Methods.* The strain of virus used was isolated from *C. tarsalis* collected in the state of Washington in 1957 and was kept at –76°C after one passage in chicks and one in suckling mice. *C. tarsalis* of the original Rocky Mountain Laboratory (RML) strain(9) were infected by feeding on 1-day-old chicks which had been inoculated with mouse-brain suspension of WEE virus. Chicks were used because they develop a more intense viremia than other laboratory animals. Before feeding mosquitoes on snakes, the mosquitoes were held at 24°C for 5-10 days to allow virus to proliferate. Wild garter snakes (*Thamnophis* spp.) were held in the laboratory at room temperature. Blood was obtained from them by snipping their tails and was tested in suckling mice for virus content. All garter snakes were tested for virus before inoculation or infective feeding.

*Results.* Three snakes were fed upon by 2, 9, and 20 infected mosquitoes, respectively. Viremia of 4 to 20 days' duration was observed (Table I). In one snake, virus was detected in a $10^{-6}$ dilution of whole blood, which was the highest dilution tested. This quantity of virus in a host is sufficent to infect mosquitoes (unpublished). All snakes died between 8th and 23rd day after mosquitoes had fed on them. There was no implication that WEE virus was responsible for death, which may have been related merely to trauma associated with bleeding.

Four snakes were injected intraperitoneally with a suspension containing $10^4$ or $10^6$ LD$_{50}$ of virus as determined by titration of the suspension in suckling mice. Virus was detected in blood of each snake. The titer of virus in one snake was at least $10^{-7}$, the highest dilution tested. Virus persisted in one snake for 36 days, at which time the snake died.

Virus isolated from blood of snakes Nos. 2, 27, 40, 94, and 50 was identified as WEE virus by neutralization test; isolates from snakes Nos. 1 and 42 were presumed to be WEE virus because of the characteristic death pattern of suckling mice. Antibody titrations in snakes surviving were not carried out.

*Summary.* 1) It has been demonstrated that garter snakes are susceptible to infection with WEE virus. This is the first evidence, to the best of our knowledge, that a virus which is an important parasite of avian and mammalian hosts can infect a cold-blooded vertebrate and cause viremia of high titer and long duration. 2) The possibility that snakes may play a role in overwintering of the virus is being investigated.

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## Spreading Factor Activity of Bacterial Chondroitinase.* (24469)

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A number of substances have been shown to increase the spread of intradermally injected indicators; however, "enzymatic spreading factor" has been applied almost exclusively to hyaluronidase(1,2). Many species of bacteria produce hyaluronidase, whose action as a spreading factor in animal tissue has been correlated with depolymerization of hyaluronic acid *in vitro*. Similarly, bacterial chondroitinase has been shown to degrade the polysaccharide chain in chondroitin sulfuric acid *in vitro*(3,4); however, spreading factor activity in animal tissue has not been described. Chondroitin sulfuric acid as well as hyaluronic acid has been isolated from skin of rabbits(5), and more recently from human gingival tissue(6). The present report is concerned with observations on spreading factor activity of chondroitinase produced by oral microaerophilic diphtheroid bacillus.

*Materials and methods.* All injections were made intradermally in albino rabbits and were given in area approximately 5 cm from spinal column on either side of animal. Bacterial culture supernates or filtrates and testicular hyaluronidase tested for spreading factor activity were administered in 0.5 ml volume consisting of 0.25 ml of test solution and 0.25 ml of 1:2 dilution of india ink in pH 6 buffer(7) and comparing area of spread with that produced by control injection of 0.85% sodium chloride, sterile broth, or test material previously heated at 100°C for 10 minutes. Animals were sacrificed, their skins removed, and areas of spread estimated by measuring diameters on underside of the dermis. To demonstrate increase in permeability of vascular connective tissue, 0.5% Evans blue was administered in 5 ml volume into marginal ear vein 3 minutes prior to intradermal injection of test material. Recordings of accumulation of dye at site of intradermal injection were made 5, 15, and 30 minutes after intradermal injection of test material. Dye accumulations were recorded as area of spread and on an arbitrary visual comparison as to intensity of color. Three strains of a chondroitinase-producing microaerophilic diphtheroid bacillus, designated as strains D2, D3, and D5, isolated originally from human gingival crevices(4), were used. A casitone-yeast extract broth containing 150 mg casitone (Difco), 50 mg yeast extract (Difco), and 50 mg sodium chloride/10 ml of medium was satisfactory for production of chondroitinase by these organisms. The pH of medium was adjusted to 7.2 before sterilization by autoclaving at 120°C for 20 minutes. Inocula were prepared from anerobic cultures maintained on sheep blood agar. Broth cultures were incubated similarly under a mixture of gas containing 95% $N_2$ and 5% $CO_2$. All cultures were incubated

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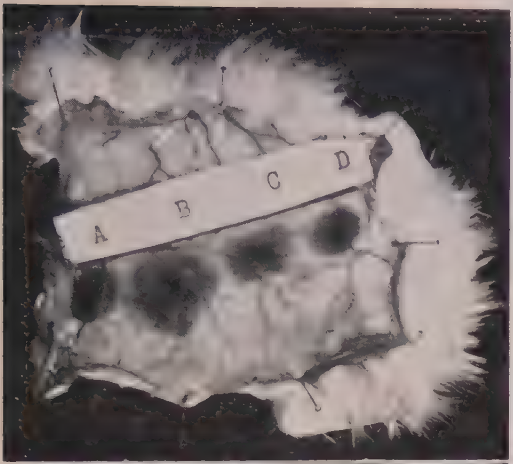
† Now at Nat. Inst. of Dental Research, N.I.H., Bethesda, Md.



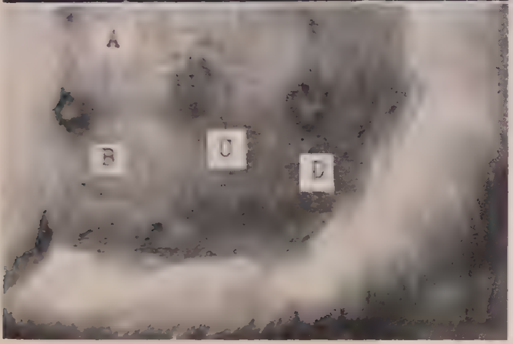


FIG. 1 (top). Spreading of india ink in rabbit skin produced by (A) sterile broth, (B) strain D3 culture supernate, (C) strain D3 heated culture supernate, and (D) 0.85% sodium chloride.

FIG. 2 (bottom). Accumulation of intravenously injected Evans blue in areas of rabbit skin produced by (A) strain D2 heated culture supernate, (B) D2 (C) D3 (D) D5 culture supernates containing chondroitinase activity.

TABLE I. Spreading of Intradermally Injected Chondroitinase and Hyaluronidase - Containing Preparations in Rabbit Skin.

| Treatment | D2* supernate | D3* supernate | Staphylococcal filtrate† |
|---|---|---|---|
| | Area of spread in $cm^2$ (1 hr) | | |
| None | 5.9 | 7.0 | 3.3 |
| Diluted 1:2 (.85% NaCl) | 3.9 | 5.9 | 3.1 |
| Diluted 1:5 (.85% NaCl) | 2.4 | 2.4 | 2.4 |
| 100°C for 10 min. | 2.4 | 2.4 | 2.4 |

* Signifies 2 strains of diphtheroid bacilli. Supernate contains approximately 25 TRU chondroitinase/ml and $< 1$ TRU hyaluronidase/ml.

† Filtrate contains approximately 40 TRU hyaluronidase/ml. No chondroitinase activity detectable.

for at least 3 days. Culture filtrates of *Staphylococcus aureus*, strain SAB2, stored at –20°C, were used as source of bacterial hyaluronidase. Testicular hyaluronidase, a product of Armour Lab., was assayed before each experiment. Chondroitinase and hyaluronidase were expressed in turbidity reducing units (TRU) (4,8), using substrates potassium chondroitin sulfate and potassium hyaluronate from human umbilical cord(7).

*Results.* Fig. 1 demonstrates typical spreading of india ink in rabbit skin by bacterial culture supernate containing 25 TRU/ml of chondroitinase. The marked increase in diffusion of india ink in the area of skin receiving the chondroitinase-containing preparation (Fig. 1B) is to be noted in comparison to that produced by any one of the 3 control injections. Table I shows results one hour after administering supernates from 2 strains of diphtheroid bacilli and from a filtrate of *S. aureus*. The chondroitinase-containing preparations effect 2 to 3 times as much spreading factor activity as that produced by staphylococcal filtrate containing 40 TRU of hyaluronidase/ml. Spreading factor activity has apparently disappeared when test materials were diluted 1:5 and as expected when preparations were heated. Heating destroyed also its *in vitro* activity.

Fig. 2 shows accumulation of intravenously injected Evans blue in areas receiving bacterial chondroitinase as compared with accumulation of dye in area receiving a heat-inactivated preparation. Accumulation of some dye in area receiving heat-inactivated preparation (Fig. 2A) can probably be explained on the basis that the medium contains certain nonspecific spreading factors such as peptones shown to increase permeability of connective tissue(9). It may be that chondroitinase in such preparations is not directly responsible for increase in vascular permeability noted. Benditt *et al.*(10) have demonstrated that purification of bull-testis hyaluronidase tends to render it incapable of increasing capillary permeability and that impurities in the extract are actually responsible for the increase in vascular permeability observed. A similar situation may exist with regard to chondroitinase-containing culture supernate. Substances other than chondroitinase could be responsible for increasing permeability of capillaries and thereby account for accumulation of some Evans blue in areas receiving heat inactivated preparations or sterile broth. The increased area of spread induced by chondroitinase-containing preparation may be due only to depolymerization of ground substance surrounding the capillary bed by the enzyme with increase in vascular permeability due to other, unknown factors. Until one has a highly purified preparation of chondroitinase, it is still questionable that the enzyme is acting directly on the capillary wall. It is certainly conceivable that the enzyme could be acting on components of vascular tissue, since the available substrate may be present(11). Attempts made to dialyze out the interfering substances in the bacterial supernate were unsuccessful.

It was of interest to observe that the action of testicular hyaluronidase and chondroitinase-containing preparations were of the same degree in increasing vascular connective tissue permeability. In both cases after intradermal injection, the bleb produced immediately flattened and accumulation of Evans blue began after about 5 minutes and showed maximum intensity of color after 30 minutes. In contrast, heat-inactivated diphtheroid supernates and heat-inactivated testicular hyaluronidase produced a bleb which remained raised almost throughout the observation period. No accumulation of dye took place in the area receiving heat-inactivated testicular hyaluronidase and only a pale blue accumulation in the area receiving heat-inactivated diphtheroid supernate. If the constituents of the culture supernate, other than chondroitinase, were responsible for the increase in vascular permeability, they are apparently different from those in bull-testis extract since these have been reported to be heat labile(10). This observation has been confirmed in our experiments since heated testicular hyaluronidase had no effect on permeability of vascular connective tissue.

*Summary and conclusions.* The finding that chondroitin sulfuric acid as well as hyaluronic acid is among the components of human gingival tissue(6), and the demonstration that microorganisms capable of breaking down this substance are present in the gingival crevice, has led to the belief that bacterial chondroitinase may be contributory to the structural breakdown of the gingival elements(4). Experiments here reported demonstrate that bacterial chondroitinase is capable of eliciting the spreading factor phenomenon in connective tissue in the same manner as bacterial and testicular hyaluronidase and may further emphasize the importance of polysaccharases in pathogenesis of periodontal disease.

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# Agents Resembling Enteroviruses Isolated in HeLa Cell Cultures from Fecal Specimens.* (24470)

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From an outbreak of respiratory illness occurring at a convalescent home for children in Washington, D. C. during summer of 1956, cytopathogenic agents with some characteristics of enteroviruses(1) were isolated in HeLa cell tissue cultures from fecal specimens. These agents did not produce cytopathic changes in monkey kidney cell cultures and also lacked pathogenicity for suckling mice and other laboratory animals. This report deals with isolation, and biologic characterization of these agents.

*Materials and methods.* Specimens: Pharyngeal, conjunctival, nasal, and anal swabs as well as stools were collected from patients and prepared as previously reported(2). All specimens were kept frozen at –20°C until used. Portions of specimens were inoculated into HeLa and rhesus monkey kidney cell cultures‡ and into 3-day-old Swiss mice. Culture media. Monkey kidney cultures were maintained in medium consisting of 98 parts Medium 199(3) and 2 parts inactivated (56°C for 30 minutes) calf serum. Maintenance medium for HeLa cell cultures consisted of 70 parts Medium 199, 25 parts tryptose phosphate broth, and 5 parts inactivated chicken serum. Maintenance medium for human embryonic fibroblasts (MAF line) was composed of 97 parts Medium 199 and 3 parts inactivated horse serum. All media contained penicillin, streptomycin, and nystatin in final concentration of 250 U, 250 $\mu$g, and 125 U/ml respectively. Stock virus pools. One-tenth ml of undiluted fluid harvested from positive tissue cultures was inoculated into tube cultures of HeLa cells. When maximal degeneration occurred the tubes were frozen at –20°C, thawed slowly, and the fluids pooled and centrifuged at 1500 rpm for 20 minutes. The supernatant fluid was decanted and aliquots stored at –50°C in screw-capped vials. Neutralization tests. Two-fold dilutions of serum (inactivated at 56°C for 30 minutes) were incubated for 2 hours at 37°C with equal volume of stock virus diluted to contain 50-100 $TCD_{50}$/inoculum. Two-tenths ml of serum-virus mixture was then inoculated into each of 2 MAF cell cultures. Final readings were made on fifth day after inoculation which was 48 to 72 hours after virus controls developed 2 to 4 plus cytopathic effects (CPE). Complete inhibition of CPE was considered evidence of neutralization. Antiserums to several strains of isolates were prepared in rabbits by intravenous injections. Seven inoculations of 1.5 ml of a stock virus pool known to contain at least $10^5$ $TCD_{50}$/ml were administered within the first 3 weeks. Two booster inoculations were given; the first, at least one month after fifth inoculation and the second, one month later. Serum was collected by cardiac puncture one week after last inoculation.

*Results.* Agents were recovered in HeLa cell cultures inoculated with fecal specimens obtained from 8 of 10 patients. Within 48 hours of specimen inoculation, there was evidence of cellular destruction. CPE and clinical disease were absent respectively in monkey kidney cultures and infant mice. Attempts in these patients to recover virus from specimens other than fecal specimens were consistently negative.

The CPE in HeLa cells could not be distinguished from that produced by poliovirus. One-tenth ml of undiluted stock virus pools produced complete degeneration within 24 hours in HeLa cells, and after inoculation of 0.5 ml there was usually one plus typical CPE within 8 hours. Limiting dilutions usually produced CPE within 72 hours. Titrations of stock virus pools in HeLa cells yielded titers of $10^{5.5}$ to $10^{7.5}$ $TCD_{50}$/ml. The prototype virus (Pett. strain) was carried through 23 passages in HeLa cells.

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‡ Cell cultures obtained from Microbiological Associates, Bethesda, Md.

MAF cultures were less sensitive for detection of virus, and with some strains showed slow progression of CPE. After several passages in MAF cells, the viruses produced rapidly progressing CPE, and this tissue could then be used for neutralization tests.

To define cell host pattern of virus isolates, 0.2 ml of stock virus pools titering $10^{5.5}$ to $10^{7.5}$ $TCD_{50}$/ml were inoculated into tube cultures of amnion, liver, intestine, conjunctiva, carcinoma (KB strain), heart and fibroblasts of human origin and into heart(4) and kidney cultures of simian origin. For these experiments the medium used for maintenance of HeLa cells was used. CPE was observed in all cell lines with the exception of monkey renal epithelium. Repeated attempts with varied amounts of inocula and progressive passage harvests of positive HeLa cultures consistently failed to provoke CPE in rhesus monkey kidney cultures. A single trial with cynomolgus monkey kidney cultures also failed to induce CPE.

No detectable disease was produced when portions of stock virus pools were inoculated by different routes into one and 3-day-old mice, adult mice, 3-day-old Syrian hamsters, and in adult guinea pigs and rabbits. From the 3-day-old mice, which were inoculated intracerebrally (i.c.) and intraperitoneally (i.p.), 2 mice were killed on fifth day after injection; 10% carcass suspensions were prepared and portions inoculated i.c. and i.p. into other 3-day-old mice. Four serial blind passages were done by this method. Mice were observed for 21 days, and none developed detectable disease. Carcass suspensions at each passage were inoculated into HeLa cells and no virus was recovered. Isolates from 5 patients were tested in this manner.

Undiluted stock virus pools were inoculated amniotically and allantoically respectively into 7- and 10-day-old embryonated hen's eggs. Fluids were harvested individually and tested for presence of hemagglutinins with human Group O and chicken erythrocytes. Fluids from each group were then pooled and passaged to HeLa cells and another set of eggs. Three and 7 serial transfers were made respectively by allantoic and amniotic routes. Of 6 isolates tested by the allantoic method and 4 isolates tested by the amniotic method, no hemagglutination was obtained and no virus was recovered in HeLa cells at any egg passage level. Also no gross lesions were seen in embryos or membranes.

No loss of virus activity was observed after treatment of virus isolates with diethyl ether in a 20% final concentration for 18 hours at 4°C. Infectivity of virus isolates was destroyed at 65°C for 30 minutes. Moreover, a significant loss of titer was noted when isolates were heated at 56°C and 62°C for 30 minutes. The agents tolerated a minimum of at least 15 cycles of freezing and thawing without any apparent loss of titer and remained viable at 4°C for at least 30 days. The agents were recovered from the original fecal specimens after storage for 2 years at –20°C. The virus passed through a Gradocol membrane of apparent pore diameter 55 mmu.

Eight stock virus pools were tested for hemagglutination activity with human type O red cells. Incubation temperatures were varied at 22°C, 37°C, and 4°C and the diluent was adjusted to pH's of 5, 6, 7, and 8. Guinea pig, less-than-3-day-old and adult chicken erythrocytes were also tested at pH 7. Each virus strain was tested more than once, and in no instance was the presence of viral hemagglutinins detected.

Initial attempts to demonstrate neutralization of even 3 $TCD_{50}$ in HeLa cultures by rabbit antiserum and human convalescent serums were unsuccessful, or at most showed only slight delay of CPE. Satisfactory neutralization tests were obtained when the tests were performed in MAF cells with pools of MAF adapted virus. Eight isolates were neutralized by 3 rabbit antiserums prepared from different strains. Also paired serums from 5 patients showed significant antibody rises against the prototype virus.

To determine serologic distinctness of the Pett. virus from known human enteroviruses, rabbit antiserum to the Pett. virus, having homologous neutralizing antibody titer of 1:160, was tested at dilution of 1:10 or 1:20 against 50-100 $TCD_{50}$ or suckling mouse $LD_{50}$

of each of the following: Coxsackie, ECHO, and poliovirus. Neutralization tests with Coxsackie Group A types 1-8, 10, 12, 14, 16, 17, and 19 were done in suckling mice, and tests with Coxsackie Group A types 9, 11, 13, 15, and 18, Coxsackie Group B types 1-5, ECHO types 1-20, and poliovirus types 1-3 were done in MAF or monkey kidney cultures. In no instance was neutralization observed.

*Discussion.* The properties of these agents in regard to size, ether resistance, temperature stability, CPE, recovery from fecal specimens, and isolation during summer would place them in the Enterovirus Group. The failure of these agents to produce CPE in monkey kidney cells and disease in laboratory animals suggested that these agents were different from enteric viruses currently reported. The inability of a prototype rabbit antiserum prepared from one of these isolates to neutralize known members of the enteroviruses added further evidence that they are not antigenically related.

Neutralization of virus isolates by 3 different rabbit antiserums would indicate that these agents are antigenically similar. The presence of specific antibody capable of neutralizing the prototype of this group in convalescent human serums would confirm their original presence in these patients. The spectrum of clinical illness associated with these agents will be described separately.

*Summary.* Agents isolated from fecal specimens in HeLa cell cultures possessed properties similar to enteric viruses. Serologic evidence indicated that these agents were not immunologically related to known members of the Enterovirus Group.

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## Blocking Urinary Electrolyte Effects of Desoxycorticosterone with Progesterone in Rats.* (24471)

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Progesterone causes an increased urinary excretion of Na in Addisonian patients treated with desoxycorticosterone (DOC) possibly by competition between the 2 steroids in the kidney; progesterone was not effective in the absence of DOC treatment(1). The present report on electrolyte effects of progesterone in rats supports these findings and shows, in addition, that DOC-like property of the steroid limits blocking efficiency at large doses.

*Method.* Male Sprague-Dawley rats (150-200 g) were adrenalectomized and maintained with sucrose cubes and tap water overnight. Approximately 24 hours post-operatively, the animals were subcutaneously injected with 2.5 ml 0.85% NaCl solution in shoulder region, and with desoxycorticosterone acetate (DCA) in 0.1 ml Mazola oil. Other animals also received subcutaneous injection of progesterone in 0.5 ml oil. A 4-hour sample of urine was collected in metabolism cages and analyzed for Na and K concentration after dilution to 25 ml with water. Mean values of Na/K ratio were determined after analyses with Beckman flame photometer, using individual or pooled samples of urine. In this method, 12 to 48 μg of DCA cause Na retention, some K loss and maximal reductions in the ratio of Na/K. Blocking activity was based on reversal of the Na/K effects of DCA, a less variable measurement than Na or K alone.

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* The author appreciates technical assistance of Misses Janet L. Wallen and Clarice A. Kieffer and helpful criticism and suggestions of Drs. V. A. Drill and C. G. Van Arman.

*Results.* Preliminary results from pooled data of separate tests showed ability of progesterone to block electrolyte effects of 12 μg DCA (Table I). Significant blocking of Na/K effects was obtained with 2.4 and 4.8 mg doses; in this method, the Na/K value of 0.94 is significant ($P<0.05$) with 18 rats. The effects of progesterone were interpreted as incomplete (*cf.* mean values of untreated controls) and resulting from simultaneous reversal of Na retention and K loss. Blocking was demonstrated with other dosage combinations of progesterone and DCA (Table II). When given alone at relatively large doses, the steroid mimicked DCA by producing low Na/K values; the change of mean Na/K between the 4.8 and 9.6 mg doses was significant ($P<0.05$). Progesterone appeared progressively less effective in reversing Na/K with increasing doses of 12, 24 and 48 μg of DCA (Table I and II). This observation is interpreted as a reduction in blocking efficiency of progesterone for it occurred with similar dosage ratios of progesterone/DCA and in the presence of approximately equal reductions in Na/K ratio with DCA. Loss of blocking efficiency is believed to result directly from manifestations of DCA-like action with large doses of the steroid.

*Discussion.* Demonstration of DCA-blocking activity by progesterone confirms the findings of Landau *et al.* in man(1). Inability of the steroid alone to elicit Na/K changes that would oppose DCA action rules out physiological antagonism as a mechanism of its inhibitory effects. As a contrast, that progesterone produced a DCA-like effect suggests an affinity for similar receptor sites in target tissues and the possibility of competition with DCA as discussed by Landau *et al.*(1). The potency of progesterone in rats, as determined by doses required for 50% block of 12 μg of DCA,[†] is estimated to be a small fraction of the activity described for steroidal lactones (2). Rosemberg also described a minimal activity for progesterone in similar animals(3). At large doses, the DCA-like property in our study and in others(4-9) appears to be an important factor that limits blocking efficiency of the compound. Thus, the steroid possesses unique electrolyte effects by inhibiting and mimicking the action of another steroid; this combination of effects in a compound is not uncommon in study with non-steroids(10,11).

TABLE I. Blocking Urinary Electrolyte Effects of Desoxycorticosterone Acetate with Progesterone.

| Treatment/rat | | | Mean urinary values | | |
|---|---|---|---|---|---|
| DCA, μg | Progesterone, mg | No. test animals | Na | K | Na/K |
| | | 65 | 6.07 | 2.80 | 2.21 |
| 12 | | 66 | 2.30 | 4.17 | .59 |
| " | 1.2 | 35 | 2.98 | 3.50 | .88 |
| " | 2.4 | 19 | 3.52 | 3.44 | 1.08 |
| " | 4.8 | 23 | 3.54 | 3.06 | 1.15 |

Na and K values = mM/l concentration after dilution of sample/rat to 25 ml.

TABLE II. Action of Progesterone in Blocking Urinary Na/K Effects of Desoxycorticosterone Acetate.

| Treatment/rat | | |
|---|---|---|
| DCA, μg | Progesterone, mg | Mean urinary Na/K* |
| 24 | | .46 |
| " | 2.4 | .89 |
| " | 4.8 | .88 |
| " | 9.6 | .97 |
| | 4.8 | 1.15 |
| 48 | | .50 |
| " | 4.8 | .69 |
| " | 9.6 | .77 |
| " | 19.2 | .75 |
| | 9.6 | .82 |

* Changes in mean Na/K value of ± 0.24 required for least significant difference ($P<0.05$).; No. test rats/treatment = 9-10.

*Summary.* Progesterone partially blocked the effects of desoxycorticosterone acetate (DCA) on the urinary ratio of Na/K in adrenalectomized rats by simultaneously reversing Na retention and K loss. Progesterone alone at large doses caused DCA-like reductions of the Na/K ratio. Tests of various dosage combinations with DCA suggest that blocking efficiency of progesterone is limited by its DCA-like property. The compound appears to be a competitive inhibitor of DCA and a unique example of a steroid with blocking and DCA-like effects in rats.

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[†] This degree of blockade corresponds to reduction of Na/K effect from 12 to 6 μg DCA, or to change in Na/K response from 0.59 to approximately 1.0.

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## Effect of Certain Flavonoids on the Pituitary-Adrenal Axis.* (24472)

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Oral administration of a number of flavonoids to animals and humans has been shown to result in the urinary excretion of a variety of hydroxy aromatic acids(1,2,3). This finding suggested that, through the medium of their metabolites, flavonoids might influence the pituitary-adrenal axis in a manner comparable to that reported for salicylic acid and related compounds(4,5). The present report concerns an investigation of this possibility with the flavonoids quercetin, dihydroquercetin, and eriodictyol.

*Methods.* Albino rats from a colony maintained in this laboratory since 1931 were used. Young rats were weaned at about 21 days of age, weighed individually, and sexes segregated. Overweight and underweight rats were discarded. When selected rats were not more than 4 weeks old they were fed for 1 to 4 weeks a basal diet with or without addition of a flavonoid. The basal diet had the following percentage composition: corn meal 73, casein 10, linseed oil cake meal 10, alfalfa meal 2, cod liver oil 3, bone ash 1.5, and sodium chloride 0.5. The quercetin was prepared by acid hydrolysis of the rhamnoside quercitrin(6), and eriodictyol was prepared from dihydroquercetin‡ by the method of Pew(7). At end of feeding period all control and experimental rats were sacrificed, body weights and thymus weights determined, and all organs examined for evidence of gross changes. Comparable experiments were performed with groups of rats which had been adrenalectomized or hypophysectomized. Adrenalectomized rats were placed on the diets immediately after operation and supplied with 0.9% sodium chloride in the drinking water. Hypophysectomized rats were placed on the diets 4 days after operation.

*Results.* The data on fresh thymus weights, expressed as per cent of body weight for rats receiving quercetin, dihydroquercetin, or eriodictyol, and their respective controls are summarized in Table I. The smaller thymus weights of flavonoid-fed rats as compared with their respective controls are clearly significant. In the case of quercetin, where the largest number of rats was used, an analysis of the individual data, with construction of

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‡ Dihydroquercetin was supplied by Oregon Forest Products Lab., Corvallis.

TABLE I. Thymus Gland Weights of Control and Flavonoid-Fed Rats.

| | Avg thymus wt expressed as % of body wt | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Body wt, g | No. of animals | Thymus | SD | No. of animals | Thymus | SD | Difference ± SE | P |
| | | Control ♀ | | | Quercetin ♀ | | | |
| 60– 79 | 22 | .309 | .028 | 14 | .276 | .037 | .0331 ± .0116 | .01 |
| 80– 99 | 16 | .301 | .038 | 9 | .271 | .037 | .0303 ± .0156 | .07 |
| 100–119 | 11 | .290 | .023 | 5 | .248 | .021 | .0413 ± .0118 | <.01 |
| | | Control ♂ | | | Quercetin ♂ | | | |
| 78–108 | 7 | .359 | .046 | 5 | .269 | .026 | .090 ± .0208 | <.01 |
| | | Control ♀ | | | Dihydroquercetin ♀ | | | |
| 67– 92 | 29 | .310 | .031 | 15 | .264 | .026 | .046 ± .0089 | <.001 |
| | | Control ♂ | | | Dihydroquercetin ♂ | | | |
| 72–100 | 7 | .359 | .046 | 8 | .282 | .028 | .077 ± .0199 | <.01 |
| | | Control ♀ | | | Eriodictyol ♀ | | | |
| 69– 84 | 20 | .316 | .033 | 4 | .243 | .027 | .073 ± .0152 | <.001 |

SD = stand. dev.; SE = stand. error of difference; P = probability that such a difference would occur by chance.

regression lines, showed that relative thymus weight gradually decreases with increase in body weight, with gradual convergence of control and experimental groups. The apparent exception to this statement, as shown in the Table for rats in weights ranging 100-119 g is probably due to the small size of the samples. We believe that the difference in thymus weights between control and flavonoid-fed rats can be shown most satisfactorily with animals weighing 50 to 80 g.

In an experiment involving 74 female and 52 male rats as controls, and 43 female and 29 male rats fed 2% quercetin, thymus gland weights were determined after fixation in 10% formalin. Involution of thymus glands was again clearly evident as in the experiments with fresh thymus weights.

The data on thymus weights summarized in Table II clearly demonstrate that feeding quercetin does not affect the weight of thymus gland in adrenalectomized or hypophysectomized young rats. No abnormalities were noted in the various organs of any animals at time of sacrifice. Removal of adrenals or pituitaries was confirmed at time of autopsy by careful examination.

The nontoxicity of quercetin(8) and dihydroquercetin(9) suggests that stress due to ingestion of these flavonoids was not a causative factor in thymus involution.

*Discussion.* Involution of the thymus gland of young rats produced by feeding quercetin, dihydroquercetin, or eriodictyol, and failure to produce involution in adrenalectomized or hypophysectomized rats suggests that physiological effects of these flavonoids are mediated, in part at least, through an action on the pituitary-adrenal axis. The antioxidant action toward epinephrine(10,11) and ascorbic acid(12), and the inhibitory action on hyaluronidase(13) possessed by flavonoids in varying degrees have been used to explain the beneficial effects of flavonoids on capillaries in a variety of experimental and clinical conditions. This mechanism of action based upon properties of the intact flavonoid molecule, and the presently suggested mechanism involving stimulation of the pituitary-adrenal axis are complementary.

TABLE II. Thymus Gland Weights of Adrenalectomized or Hypophysectomized Female Rats Fed Control or Quercetin-Containing Diet.

| | Control diet | | | Quercetin-containing diet | | |
|---|---|---|---|---|---|---|
| | No. of rats | Avg body wt, g | Thymus, % of body wt | No. of rats | Avg body wt, g | Thymus, % of body wt |
| Adrenalectomized | 10 | 64 | .33 | 11 | 68.5 | .32 |
| Hypophysectomized | 9 | 56 | .28 | 9 | 53.5 | .28 |

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# Lipide Changes in Chylomicra and Subnatant Fractions of Rat Lymph During Cholesterol Absorption.* (24473)

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The effects of sodium taurocholate, oleic acid, and albumin, singly and in combinations, on absorption of exogenous and endogenous cholesterol in the rat were reported by Vahouny *et al.*(1,2,3). It was shown that lipide composition of lymph during absorption was related to composition of the test emulsion administered. Laurell(4) has reported the percentage distribution of lipides in chylomicra and lipoproteins of the lymph of rats which were fed one type of emulsion. Byers and Friedman(5) reported that dietary cholesterol travelled almost completely in the chylomicron fraction in the lymph of rats which were fed cholesterol in olive oil. Jobst and Schetter(6) isolated chylomicrons from chylic ascites and chylic pleural discharge of a patient suffering from Whipple's disease and found no differences in these types of chylomicra. In the experiment described below the lipide changes in the chylomicron and subnatant fractions of the lymph of rats which were administered various test emulsions were determined in order to evaluate more clearly the roles played by chylomicra and lipoproteins in lymph lipide transport.

*Methods.* Adult male rats of the Carworth Farms strain, weighing between 175-250 g were employed. Collection of lymph, preparation and intragastric administration of the test emulsions were as described by Vahouny *et al.*(1). The basic emulsion contained 3 ml of isotonic saline solution and 50 mg of albumin. The test emulsions contained, in addition to the basic emulsion, amounts of cholesterol, oleic acid, triolein and sodium taurocholate shown in Table I. Ten groups of 3 or 4 rats each were used and they are numbered in the tables corresponding to the number of the emulsion shown in Table I which they received. Lymph was collected

TABLE I. Composition of Test Emulsions.

| | Emulsion No. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9* | 10 |
| Albumin, 50 mg | + | + | + | + | + | + | + | + | + | + |
| Sodium taurocholate, 287.4 mg | | | | | + | + | + | + | + | + |
| Oleic acid, 290.9 mg | | | + | + | | | + | + | + | |
| Triolein, 303.9 mg | | | | | | | | | | + |
| Cholesterol, 50 mg | | + | | + | | + | | + | + | + |
| .9% saline to 3 ml | + | + | + | + | + | + | + | + | + | + |

* Oleic acid was increased to 878.7 mg.

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TABLE II. Changes in Neutral Fat and Phospholipide in Lymph Fractions during Absorption.

| Group and emulsion | 2 | | 3 | | 4 | | 7 | | 8 | | 9 | | 10 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | C* | S | C | S | C | S | C | S | C | S | C | S | C | S |
| Neutral fat | 6 | 30 | 37 | 64 | 47 | 54 | 58 | 46 | 80 | 46 | 117 | 74 | 108 | 103 |
| Stand. error | ±2 | ±4 | ±10 | ±14 | ±3 | ±7 | ±11 | ±6 | ±15 | ±9 | ±34 | ±6 | ±6 | ±5 |
| ''Extra'' neut. fat | | | 31 | 34 | 41 | 24 | 52 | 16 | 74 | 16 | 111 | 44 | 102 | 73 |
| Phospholipide | 1.6 | 3.2 | 2.6 | 8.6 | 2.3 | 8.6 | 3.6 | 6.6 | 7.5 | 7.8 | 12.2 | 6.4 | 5.3 | 7.7 |
| Stand. error | ±.3 | ±.6 | ±.8 | ±2.4 | ±.8 | ±.8 | ±.5 | ±1.7 | ±1.7 | ±.6 | ±.2 | ±1.4 | ±.9 | ±.9 |
| ''Extra'' phospholipide | | | 1.0 | 5.4 | .7 | 5.4 | 2. | 3.4 | 5.9 | 4.6 | 10.6 | 3.2 | 3.7 | 4.5 |

* C = Chylomicron; S = Subnatant.

All values are in mg/12 hr lymph collection.

Stand. errors of means were calculated using the formula: $S.E. = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

for 12 hrs after administration of the emulsion. From a portion of each 12 hr lymph sample the chylomicron and subnatant fractions were separated at 10,300 g for 1 hour, as described by Lindgren *et al.*(7). The samples of chylomicrons were washed twice with saline of density 1.006. Alcohol-acetone (1-1) extracts of each sample of whole lymph, and of each chylomicron, and subnatant fraction were prepared(1) and analyzed for total lipide(8), free and total cholesterol(9) and phospholipide(1). Neutral fat was calculated by difference(1). The average values for all fractions are given in the tables as mg per 12 hr lymph sample. "Extra" cholesterol, neutral fat, and phospholipide refer to the increase in these fractions over the appropriate control levels. Group 2 was used as control for neutral fat and phospholipide shown in Table II. Group 3 was used as control for Group 4, Group 5 for Group 6, and Group 7 for Groups 8, 9, and 10 in evaluation of changes in cholesterol content (Table III). In all groups the sum of the lipide fractions in the chylomicron and subnatant fractions agreed closely with the levels in the parent 12 hr lymph samples. These lymph data are not included in the tables but they indicated satisfactory recovery of the lipides in the fractions. Emulsion 1 gave the same levels and distribution of lipides in the chylomicron and subnatant fractions as Emulsion 2 and it has been omitted from the tables.

*Results.* The data on effects of test emulsions on neutral fat and phospholipide of the lymph fractions are shown in Table II. Inclusion of oleic acid alone (Group 3) produced comparable increases in neutral fat in both chylomicron and subnatant fractions. When cholesterol or taurocholate was also included (Groups 4 and 7) with oleic acid the magnitude of the increases in neutral fat was the same as with oleic acid alone but there was a shift in distribution so that the major part of the increase was in the chylomicron fraction. Administration of both cholesterol and taurocholate with oleic acid produced a further increase in lymph neutral fat which was entirely in the chylomicron fraction. Tripling the oleic acid gave additional increments in neutral fat of comparable magnitude in both fractions. Triolein gave twice as much "extra" neutral fat as a comparable amount of oleic acid (Groups 8 and 10).

Relative increases and distribution of phospholipide between the fractions did not follow the pattern for neutral fat. There was a significant and relatively constant increase (3-5 mg) in the subnatant fraction in all groups. There were small insignificant increases in the chylomicron fraction of Groups 3 and 4, those in which highly significant increases occurred in neutral fat. Relative proportions of phospholipide in the 2 fractions were different from neutral fat in Groups 7, 8, 9, and 10.

Changes in cholesterol content of the lymph fractions are shown in Table III. Comparison of Groups 3 and 4 shows that addition of cholesterol to the oleic acid emulsion gave increases in both fractions with 76% of the total increase occurring in the subnatant frac-

TABLE III. Changes in Cholesterol in Lymph Fractions during Absorption.

| Group and emulsion | 3 |  | 4 |  | 5 |  | 6 |  | 7 |  | 8 |  | 9 |  | 10 |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | C | S | C | S | C | S | C | S | C | S | C | S | C | S | C | S |
| Cholesterol | 1.4* | 3.9 | 3.1 | 9.4 | .4 | 5.9 | .9 | 7.6 | 1.4 | 3.2 | 3.9 | 7.2 | 8.6 | 8.6 | 1.5 | 5.3 |
| Stand. error | ±.2† | ±1.3 | ±.9 | ±1.9 | ±.2 | ±1.0 | ±.2 | ±.9 | ±.3 | ±.7 | ±.4 | ±.9 | ±.7 | ±.6 | ±.9 | ±2.5 |
| "Extra" cholesterol |  |  | 1.7 | 5.5 |  |  | .5 | 1.7 |  |  | 2.5 | 4.0 | 7.2 | 5.4 | .1 | 2.1 |

* All values are in mg/12 hr lymph collection.

† Stand. errors of the means were calculated using the formula: $\text{S.E.} = \sqrt{\dfrac{\Sigma d^2}{n(n-1)}}$.

tion. Administration of cholesterol (Group 6) with taurocholate gave a smaller increase than with oleic acid but 77% of the increase was in the subnatant fraction. Likewise in Groups 8 and 10 the major part of the increase was in the subnatant fraction. Only in Group 9 was the increase greater in the chylomicron fraction; here the oleic acid was 3 times that in Group 8. The differences between Group 8 and 10 indicate that triolein gave only one-third the increase in cholesterol given by an equivalent quantity of oleic acid.

The percentage composition of the total lipides of the chylomicron and subnatant fractions is shown in Table IV. It is quite evident that the lipide mixture in the 2 fractions differed for each emulsion and that the chylomicron fraction was different for each emulsion except for Groups 3 and 7. Also there was considerable variation in composition of the subnatant fractions but Groups 2 and 9, 3 and 7, and 4 and 8 were similar. In all chylomicron fractions more than half of the lipide mixture was neutral fat. This was also true for the subnatant fractions except Group 6.

*Discussion.* The present experiment clearly demonstrates that the increase in lymph lipides which occurs during fat absorption is due to increases in both chylomicron lipides and those of the lipoproteins of the subnatant fractions. Thus the chylomicrons are not the sole means for lymphatic transport of either absorbed fatty acid or cholesterol. In most of the groups the chylomicrons carried the major part of the increase in neutral fat. However, in Group 10, which received a triglyceride, there was 92% more neutral fat in the lymph than in Group 8 which received an equivalent amount of fatty acid but 67% of the increase was carried in the subnatant fraction. Absorbed cholesterol was carried principally in the subnatant fraction except when a relatively large amount of free fatty acid (Group 9) was administered. In agreement with earlier findings the data show that free fatty acid may be a limiting factor in cholesterol absorption and that the fatty acids of triglycerides are not quantitatively equivalent to free fatty acids in promoting cholesterol absorption.

TABLE IV. Lipid Composition of Chylomicron and Subnatant Fractions.

| Group and emulsion | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | % | | | | |
| | | | | Chylomicron fractions | | | | | |
| Neutral fat | 74.4 | 89.1 | 86.9 | 57.9 | 80.9 | 91.1 | 85.2 | 82.6 | 93.0 |
| Phospholipide | 19.5 | 6.3 | 4.2 | Trace | Trace | 5.6 | 8.2 | 8.6 | 4.6 |
| Free cholesterol | 2.5 | 1.2 | 1.3 | 10.5 | 2.8 | .8 | 1.0 | .9 | 1.5 |
| Cholesterol esters | 3.6 | 3.4 | 7.6 | 31.6 | 16.7 | 2.5 | 5.6 | 7.9 | .9 |
| | | | | Subnatant fractions | | | | | |
| Neutral fat | 75.5 | 81.8 | 70.2 | 61.7 | 32.5 | 80.4 | 70.6 | 78.9 | 86.6 |
| Phospholipide | 8.8 | 11.0 | 11.2 | 16.5 | 24.9 | 11.4 | 12.1 | 6.8 | 6.6 |
| Free cholesterol | 3.9 | 1.5 | 3.0 | 4.2 | 5.6 | 1.5 | 2.3 | 1.7 | 1.5 |
| Cholesterol esters | 11.8 | 5.7 | 15.6 | 17.6 | 37.0 | 6.7 | 15.0 | 12.6 | 5.3 |

*Summary.* Changes in distribution of neutral fat, phospholipide, and cholesterol between chylomicron and subnatant fractions of rat lymph have been produced by feeding emulsions containing cholesterol, fatty acid, triglyceride, and taurocholate in various combinations. Increases in neutral fat were carried largely in the chylomicron fraction while increases in cholesterol were carried largely in the subnatant fraction. The emulsions also produced changes in percentage composition of the lipide mixture in each fraction.

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# Immunological and Enzymological Specificity of Antibodies Produced by Injection of Purified Acetylcholinesterase. (24474)

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This paper is concerned with results of experiments in which an attempt was made to produce specific inhibitory antibodies against a particular type of cholinesterase enzyme. Reports have been published in which specific inhibitory antibodies have been obtained against certain enzymes. Mansour *et al.*(1) immunized roosters with rabbit muscle lactic dehydrogenase and obtained sera which markedly inhibited the activity of this enzyme but had no effect on lactic dehydrogenase of *Schistosoma mansoni* or *Schistosoma japonicum.* Conversely, it was shown(2) that antisera to the lactic dehydrogenase of *Schistosoma mansoni* markedly inhibited the activity of this enzyme and that from *Schistosoma japonicum* and *haematobium* but not the lactic dehydrogenase from rabbit muscle. The antigenicity of a cholinesterase enzyme had been suggested by Holland(3). He reported that sera from rabbits immunized with purified ox erythrocyte cholinesterase inhibited

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hydrolysis of acetylcholine by fresh ox erythrocytes about 50% and caused hemolysis of erythrocytes in the complement hemolysis test. Hydrolysis of acetylcholine by the purified enzyme was inhibited about 25% with antisera. Therefore, since it was anticipated that animals immunized with a cholinesterase enzyme would produce antibodies that would be specifically inhibitory for a certain species or particular type of cholinesterase, rabbits and dogs were injected intravenously with purified bovine erythrocyte acetylcholinesterase.

*Materials and methods.* Five male rabbits, weighing about 3 kg each, and 3 female dogs, weighing about 10 kg each, were injected intravenously every other day total 10 injections with purified acetylcholinesterase or gelatin used to stabilize the enzyme. The purified bovine erythrocyte acetylcholinesterase and the gelatin were obtained from Winthrop Laboratories. Three rabbits each received 20 mg of acetylcholinesterase in 1 ml of 0.9% sodium chloride solution (saline) and 2 rabbits each received equivalent amounts of gelatin (6.7 mg in 1 ml saline). This was the amount of gelatin present in the injection of acetylcholinesterase. Two dogs received 30 mg of acetylcholinesterase in 1.5 ml of saline and one dog received 10 mg of gelatin in 1.5 ml of saline. Sterile technic was used whenever possible. All animals received intermittent intramuscular injections of 50,000-100,000 units of penicillin. Forty days after last injection, an extra injection of 50 mg of acetylcholinesterase or 20 mg of gelatin was administered/rabbit. One rabbit (#4) that received acetylcholinesterase died within 24 hours after this injection. Blood, 10-20 ml, was drawn aseptically from 12 hour fasted animals before, during and after the series of injections. Heparin prevented clotting. Blood samples were centrifuged 15 minutes at 3,000 rpm. (International Centrifuge) and the plasmas removed. Plasma samples were tested for their effect on purified acetylcholinesterase activity using the manometric technic, and for presence of circulating antibodies using the ring precipitin test. Activity of acetylcholinesterase was determined in the presence and absence of plasma samples. Acetylcholine and acetyl-beta-methylcholine (initial concentrations of 0.01 M) were used as substrates. The purified acetylcholinesterase, diluted in 0.1% gelatin solution for stabilization purposes, could be stored in refrigerator for several weeks without appreciable loss in activity. The quantity of enzyme used/flask was 0.05 mg contained in 0.5 ml of gelatin solution. Augustinsson's buffer (0.015 M NaCl, 0.04 M $MgCl_2$, 0.025 M $NaHCO_3$, final concentrations) was used throughout manometric assays(4). When testing for inhibitor activity 0.1 ml of plasma was used/flask. An amount of plasma greater than 0.1 ml added to purified enzyme resulted in total amount of cholinesterase which was too great to maintain an optimum substrate concentration for any length of time. It was necessary to use plasma control flasks since there was appreciable activity in the plasma using either acetylcholine or acetyl-beta-methylcholine. The cholinesterase in rabbit plasma corresponds to the "true" cholinesterase(4). The final volume of solution in each flask was 3 ml. The flasks were equilibrated with 5% $CO_2$ and 95% $N_2$ at 37°C. Activity of purified acetylcholinesterase is expressed as number of microliters of $CO_2$ evolved from the bicarbonate buffer/hour/0.05 mg of purified enzyme. The effect of plasma on purified acetylcholinesterase activity was calculated from the resultant activity obtained by subtracting activity in control flasks containing only plasma from activity obtained in flasks containing both plasma and purified enzyme. Using the standard ring technic(5), the precipitin test was usually carried out concomitantly with cholinesterase assays. The ring test consisted in layering 0.2 ml of antigen, the purified acetylcholinesterase in 0.9% sodium chloride solution, over 0.2 ml of clear plasma. Formation of a just detectable cloudy ring at the interface of the 2 solutions within ½ hour was considered a positive reaction. Dilutions of the antigen ranged from 1:1,000 (1 mg/ml saline) to 1:50,000. In an identical manner gelatin was layered over the plasma. The 1:1,000 dilution of gelatin contained 0.33 mg of gelatin in 1 ml of saline. This was the amount con-

TABLE I. Effect of Plasma from Rabbits and Dogs Injected with Purified Acetylcholinesterase and/or Gelatin on *In Vitro* Activity of Purified Acetylcholinesterase (AChE).

| | Activity of acetylcholinesterase (mm³ $CO_2$/hr/0.05 mg enzyme) | | | | | |
|---|---|---|---|---|---|---|
| | Acetylcholine (.01 M) | | | Acetyl-beta-methylcholine (.01 M) | | |
| Animal No. | AChE alone | AChE in presence of plasma* | % inhibition | AChE alone | AChE in presence of plasma* | % inhibition |
| Rabbits | | | | | | |
| 1† B‡ | | | | | | |
| A (10) | 168 | 173 | −3 | 55 | 46 | 16 |
| 2 B | | | | | | |
| A (9) | 176 | 172 | 2 | 60 | 49 | 18 |
| 3 B | 182 | 172 | 6 | 66 | 54 | 18 |
| A (0) | 162 | 162 | 0 | 54 | 52 | 4 |
| A (7) | 178 | 180 | −1 | 62 | 51 | 18 |
| 4 B | 175 | 173 | 1 | 67 | 50 | 25 |
| A (9) | 175 | 175 | 0 | 56 | 46 | 18 |
| A (18) | 172 | 171 | <1 | 53 | 42 | 21 |
| 5 B | | | | | | |
| A (8) | 170 | 163 | 4 | 57 | 42 | 26 |
| A (17) | 176 | 170 | 2 | 57 | 48 | 16 |
| Dogs | | | | | | |
| 1 B | 168 | 162 | 4 | 59 | 48 | 19 |
| A | | | | | | |
| 2 B | 173 | 156 | 10 | 63 | 57 | 10 |
| A (7) | 172 | 163 | 5 | 64 | 52 | 19 |
| 3 B | 172 | 169 | 2 | 50 | 46 | 8 |
| A (3) | 176 | 161 | 9 | 58 | 49 | 16 |
| A (12) | 162 | 157 | 3 | 54 | 49 | 9 |

* AChE in presence of plasma is the resultant activity of AChE plus plasma minus activity of plasma alone.

† Rabbits 1 and 2, and Dog 1 received gelatin. Rabbits 3, 4, 5 and Dogs 2 and 3 received acetylcholinesterase.

‡ B = Activity before, and A = Activity after series of inj. of AChE or gelatin.

Numbers in parentheses are days after last inj. of antigen on which blood was drawn. (0) refers to a sample drawn during series of injections.

tained in the 1:1,000 dilution of purified acetylcholinesterase.

*Results.* The effect of pre- and post-injection plasmas from rabbits and dogs on activity of purified acetylcholinesterase are given in Table I. Plasmas from animals injected with acetylcholinesterase, when compared with plasmas from control animals or with plasmas from the same animals before injection with the enzyme, showed no significant increase in inhibition of acetylcholinesterase. Plasmas before the series of injections and control animal plasmas as well as post injection plasmas, all exhibited a "natural" inhibition of the enzyme ranging from 10 to 25% using acetyl-beta-methylcholine as substrate for the purified acetylcholinesterase. The "natural" inhibition exhibited by plasmas when acetylcholine was used as substrate was less. There was no greater inhibitory activity in plasmas of rabbits tested 13 days after the booster injections than before this injection.

Plasmas from rabbits injected with acetylcholinesterase exhibited a positive ring precipitin reaction with a titer up to 1:10,000 using acetylcholinesterase as the antigen. There was no precipitin reaction using gelatin as the antigen in the precipitin test. Plasmas of all dogs and the rabbits injected with gelatin exhibited negative precipitin reactions against enzyme and gelatin. A 1:20,000 titer was obtained with plasmas of rabbits 13 days after the booster injection of acetylcholinesterase.

*Discussion.* Several explanations can be offered for the presence in plasmas of antibodies which caused a positive ring test and absence of inhibitory activity toward the purified enzyme. First, one could assume that purified acetylcholinesterase was not antigenic. Antibodies produced from injection of purified acetylcholinesterase could have resulted from foreign proteins present in the preparation. The activity/unit weight of purified enzyme preparation varies from batch to batch; therefore more or less extraneous proteins could have been present. Secondly, one could assume that the purified acetylcholinesterase was antigenic. Studies have indicated that the molecular weight of acetylcholinesterase of electric eel is around 3,000,000(6). However, it has been proposed, based on irradiation data(7), that this macromolecule is composed of 30-50 enzymatically active units, each with a molecular weight around 105,000. These should be large enough to be antigenic. On this basis, it could be postulated that there might be a further dissociation of this subunit into an antigenic non-enzymatically active part and an enzymatically active non-antigenic part. We found that incubation for one hour at 37°C or refrigeration overnight of the purified enzyme with a rabbit's plasma, which had a ring precipitin titer of 1:10,000, produced a flocculent precipitate. In either case the activity of the purified acetylcholinesterase in the supernatant fluid remaining after such incubation was unchanged from that in the original enzyme solution when corrected for activity of added plasma. Therefore, no active enzyme was removed from solution in the precipitate. Antibodies produced under experimental conditions reported here must either have resulted from contaminating proteins in the acetylcholinesterase preparation or else antibodies produced against the acetylcholinesterase enzyme did not inhibit its enzymatic activity. On the basis of the assumption that this cholinesterase enzyme is not antigenic, experiments are presently being conducted to test this procedure as a method for further purification of this enzyme; *i.e.*, precipitation of extraneous proteins in the purified acetylcholinesterase preparation using the gamma globulin fraction of immunized rabbit's sera.

Lack of agreement between results presented here and those of Holland(3) is difficult to explain. In the report by Holland, data using purified ox erythrocyte cholinesterase as the enzyme in the manometric studies were not given. However, it was stated that antisera from the immunized rabbits inhibited the purified cholinesterase preparations 25 to 30%. This degree of inhibition was approximately the same as that obtained in experiments reported here using normal plasma and purified enzyme with acetyl-beta-methylcholine as substrate. The presence of "natural" inhibitors of cholinesterase enzymes in serum has been demonstrated by Karczmar and Koppanyi(8). These workers reported that serum, diluted 1-4, inhibited acetylcholinesterase and cholinesterase in various tissue mixtures as much as 60%. It should be pointed out that in the present experiments there were no detectable antibodies in any of the dog plasmas. However, up to 20% inhibition of the purified enzyme preparation was obtained in several cases using either pre- or post-injection plasmas when acetyl-beta-methylcholine was the substrate, and up to 10% inhibition was obtained using acetylcholine as the substrate.

*Summary.* 1) Intravenous injection of purified bovine erythrocyte acetylcholinesterase caused production of circulating antibodies in rabbits but not dogs. 2) Pre- and post-injection plasmas of all animals caused up to 25% inhibition of the purified acetylcholinesterase using acetyl-beta-methylcholine and up to 10% inhibition using acetylcholine as substrate.

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# Pregnancy and Liver Regeneration in Partially Hepatectomized Rats.

(24475)

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Because of the rapid proliferative changes involved in both pregnancy and liver regeneration, a study was undertaken of the interrelationship between these 2 phenomena in partially hepatectomized rats. According to an earlier report by Paschkis, Cantarow and Stasney(1), rats which were partially hepatectomized on the eighth or seventeenth day of gestation and sacrificed on the twentieth day of pregnancy suffered severe fetal damage. The latter ranged up to complete resorption of all fetuses in several cases and "liver regeneration was identical in nonpregnant and pregnant animals."

*Methods.* Mature female rats of Sprague-Dawley strain were employed. Virgin animals were exposed to fertile males for 7 days, then these females and nonpregnant controls were partially hepatectomized under ether anesthesia according to the procedure of Higgins and Anderson(2). The excised portions were dried to constant weight in an oven at 100°C. The rats were maintained in individual cages and administered water and powdered Purina Fox Chow *ad. lib.* They were sacrificed (ether) after an interval of 10.5 days and the livers removed and dried. The uteri of the gravid animals were closely examined at necropsy for extent of pregnancy or resorption; the data from the "negatives" were discarded. As described previously, the amount of liver regenerated or the increment was calculated from dry weights by subtracting the amount of tissue removed at surgery multiplied by 0.46 from the weight of the entire liver at necropsy(3).

*Results.* Body weights and liver increments for groups of controls, pregnant rats and those showing fetal resorption as well as the corresponding Fisher $t$ values are given in Table I. The gravid animals ranged mainly from middle to late pregnancy, several being close to term when sacrificed. Microscopic examination of representative small liver sections removed at necropsy from the pregnant rats showed no remarkable pathology over those from the controls (hematoxylin-eosin stained).

*Discussion.* A marked increase in extent of liver regeneration in partially hepatectomized rats was caused by pregnancy. Presumably, one or more factors elaborated systemically during pregnancy predispose to such increased regenerative activity. A smaller but statistically significant elevation was also apparent in animals displaying resorption of the fetuses. In this regard, the liver increments were quite high for 5 of the 11 rats which exhibited recent or active resorption as compared to the remainder where this process was almost complete. The extensive resorptive changes should not be construed as due to liver regeneration as such but to the extreme surgery involved, numerous other types of which can lead to such damage.

*Summary.* The extent of liver regeneration in partially hepatectomized rats is markedly elevated by pregnancy. A lower but significant increase over nonpregnant controls was also evident with those animals displaying fetal resorption, especially where the latter was in active progress.

TABLE I. Body Weight and Liver Increment Data for Partially Hepatectomized Control and Pregnant Rats.

| | No. of animals | Body wt, g* | | Liver increment, g* | t |
|---|---|---|---|---|---|
| | | Initial | At necropsy | | |
| Controls (nonpregnant) | 25 | 205 ± 3.2 | 207 ± 3.6 | 1.524 ± .054 | |
| Pregnant | 16 | 215 ± 4.0 | 232 ± 5.7 | 1.933 ± .067 | 4.75† |
| Rats displaying fetal resorption | 11 | 215 ± 3.5 | 219 ± 4.8 | 1.740 ± .099 | 2.06† |

* Stand. error follows each ± sign.

† $P < .05$.

This study was aided by grant from Toni Division of Gillette Co.

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# Failure of Deoxyribonucleic Acid to Effect Somatic Transformation in the Rat.[*] (24476)

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It is well established that isolated deoxyribonucleic acid (DNA) can produce heritable changes in several species of bacteria. This was first demonstrated by Avery *et al.*(1), who found that DNA extracted from a smooth (encapsulated) strain of *Pneumococcus* would transform rough (unencapsulated) cells into smooth cells. The capsule of the newly modified cells was serologically the same as that of the strain from which the DNA had been derived, and the conversion could be brought about in a rough pneumococcal strain which had not been observed to mutate spontaneously. In addition, the transformed cells propagated indefinitely as a smooth strain without further treatment with DNA. The pneumococcal DNA preparation had performed 2 functions usually attributed to genes: it had induced a specific heritable property, and had initiated its own reduplication. Subsequently other investigators demonstrated ability of isolated DNA to effect transformations in other species of bacteria, *viz. Hemophilus influenzae, Meningococcus, Escherichia coli,* and the crown-gall organism *Agrobacterium*(2). Benoit *et al*(3,4) recently have reported that somatic changes are produced in one strain of ducks by injection of DNA derived from another strain, and that the changes were transmitted to progeny of the treated birds. The present paper describes an attempt to produce a similar transformation in mammals. This work was stimulated by the prospect that a successful result might have application to the treatment of genetically determined diseases in man.

*Material and methods. Preparation of DNA.* DNA was isolated from thymuses and spleens of rats by a modication of the method used by Benoit *et al.* (personal communication) to prepare DNA from duck erythrocytes and testicular tissue.[†] Adult rats were killed, and the spleen and thymus were immediately removed and frozen over dry ice. All further steps in isolation of DNA were carried out in a cold room at 0°C or in a refrigerated centrifuge. The following solutions were used in the preparation of DNA: I. Versene-fluoride: 18.7 g disodium versenate and 1.85 g sodium fluoride in 1000 ml water, adjusted to pH 7.0 ± 0.1. II. Fluoride-chloride: 58.0 g sodium chloride, 1.85 g sodium fluoride, and 8.0 g disodium versenate in 1000 ml water, adjusted to pH 7.0 ± 0.2. III. Versene: 3.75 g disodium versenate in 1000 ml water, adjusted to pH 7.0 ± 0.2. IV. Phosphate buffer, 2.5 M, pH 8.4. V. Sodium chloride solution, 0.85%. 100 g of frozen rat spleen and thymus was minced through a meat grinder and homogenized with 300 ml versene-fluoride solution in a Waring Blender for 5 minutes. The suspension was then centrifuged at 2500 rpm for 30 minutes, the supernatant liquid was discarded, and the sedimented nuclear fragments were collected. Homogenization and centrifugation were repeated as above 5 times. The nuclear fragments then were suspended in 400 ml fluoride-chloride solution and stirred overnight.

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[*] This work supported by Ford Fn.

[†] This modification was devised by Dr. Matthew Meselson, Calif. Inst. of Tech.

The suspension was centrifuged for 30 minutes at 30,000 g. The supernatant was separated and added to 20 ml 2.5 M phosphate buffer, and the mixture saturated with sodium chloride. The sediment from centrifugation was resuspended in 100 ml fluoride-chloride, stirred overnight, then centrifuged for 30 minutes at 30,000 g. To the supernatant, 5 ml 2.5 M phosphate buffer was added, and the solution was saturated with sodium chloride. The 2 salt-saturated solutions were combined, stirred for 3 days, then centrifuged at 80,000 g for 1 hour. The sediment was discarded and the supernatant filtered if not clear. The filtrate then was poured into 2 volumes of 95% ethanol with stirring and the white fibrous clot was collected on a wooden paddle. Excess alcohol was pressed out of the clot, which was redissolved in 100 ml versene solution by stirring overnight or longer. The resulting solution was centrifuged for 30 minutes at 30,000 g, the sediment was discarded, and the alcoholic precipitation was repeated. The final clot of DNA was dissolved with stirring in a minimal volume of 0.85% sodium chloride solution, and the resultant solution of DNA was dialysed against 0.85% sodium chloride solution to remove any traces of versene or fluoride. Approximate concentration of DNA in the final preparation was determined spectrophotometrically at 260 m, and the DNA solution was stored in the refrigerator until used. *Animals.* Three strains of rats were used: Addis-Slonaker, in which the animals always have pink eyes and white hair; Long-Evans, a hooded strain in which animals have black eyes, black heads and other patches of black hair on the coat; and the CNH mutant strain of hooded rat,‡ in which homozygous recessive individuals have a constitutional nonhemolytic hyperbilirubinemia. Homozygous recessive animals of the CNH strain become jaundiced within a few hours of birth, and the serum bilirubin level is always elevated. They are less hardy than their non-jaundiced (*i.e.* heterozygous or homozygous normal) litter mates, grow more slowly, and either fail to breed or produce small litters. Many of them are ataxic, possibly due to kernicterus. The hyperbilirubinemia of these rats has been shown to be due to a congenital absence in the liver of an enzyme, glucuronyl transferase, which is required for normal conversion of relatively insoluble bilirubin into soluble bilirubin glucuronide, which is excreted into the intestinal tract by way of the bile(5). Either subcutaneous or intraperitoneal injections of DNA were given to rats daily, starting on the first day of life. The solutions were highly viscous and were given in as high a concentration as could be injected through a 26 gauge hypodermic needle. The volume of solution injected was increased at intervals as the animals grew, and generally was the maximum which the animals seemed able to tolerate.

*Results. Attempts to produce pigmentary changes in white rats.* Nine newborn Addis-Slonaker rats, all litter mates, were selected for subcutaneous injection of DNA. Four rats were each given 17 injections of DNA derived from Long-Evans rats during the first 19 days of life, each rat receiving a total of 78 mg DNA. Three litter mates were each given 17 injections of DNA derived from Addis-Slonaker rats (their own strain) during the first 19 days of life, each rat receiving a total of 72 mg DNA. The remaining 2 litter mates served as uninjected controls. All developed normally, and the injected rats showed no adverse effect produced by DNA. None of the injected rats showed any change in normal white hair color or pink eyes.

Ten newborn Addis-Slonaker rats were used for intraperitoneal injection of DNA. Five were given Long-Evans DNA; 2 of these died on the 12th and 13th days, presumably as a result of the intraperitoneal injections. The 3 survivors each received a total of 18 injections of DNA in the first 22 days of life, totalling 57 mg/animal. Five litter mates served as uninjected controls. The 3 injected rats, as well as the controls, reached adult life, and none showed any pigmentary change.

Two rats which had been injected subcutaneously with Long-Evans DNA were subsequently bred with 2 rats which had been injected intraperitoneally with Long-Evans

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‡ Supplied through the courtesy of Prof. E. R. Dempster and Mr. H. Hibbard, Dept. of Genetics, School of Agriculture, Univ. of California, Berkeley.

DNA. Both matings resulted in normal litters, and rats of the $F_1$ generations showed no change from normal pigmentation of the Addis-Slonaker rat.

*Attempts to relieve hyperbilirubinemia with injections of "normal"* DNA. Four jaundiced newborn litter mates of the CNH strain were given 16 subcutaneous injections of DNA derived from Addis-Slonaker rats, in which hyperbilirubinemia due to absence of glucuronyl transferase is unknown. Each rat received a total of 45 mg DNA during the first 18 days of life. These rats grew at a rate comparable to that shown by other jaundiced rats in the colony, and 2 developed obvious ataxia. No decrease in degree of jaundice was noted. Serum bilirubin levels were determined on these rats at the ages of 5, 10 and 15 weeks, by the method of Malloy and Evelyn(6) as modified by Kaplan and del Carmen(7). Serum bilirubin levels in each determination varied between 5 and 10 mg/100 ml, as did those of all tested uninjected jaundiced rats in the colony.

Two attempts were made to inject Addis-Slonaker DNA intraperitoneally into jaundiced newborn CNH rats. These rats tolerated intraperitoneal injections poorly, and on both attempts no rat survived long enough for serum bilirubin determinations to be done. Two animals survived a total dose of 47 mg DNA each in 16 daily injections, and 3 survived a total dose of 16 mg each given in 10 injections. These rats remained markedly jaundiced until they died at between 3 and 5 weeks of age.

Other attempts were made to test the possibility of influencing the embryo by injections of foreign DNA into the pregnant mother. Two jaundiced female CNH rats were successfully mated with a jaundiced CNH male. Since both parents were known to be homozygous recessives, all offspring produced could be expected to be jaundiced. One female rat was given 10 daily subcutaneous injections of Addis-Slonaker DNA during the first 10 days after insemination, a total dose of 300 mg DNA; she produced a litter of 5 rats, all of which were jaundiced. All 5 failed to thrive and died early. The oldest survivor died at the age of 4 weeks jaundiced and ataxic. The second female was given 10 daily intraperitoneal injections of Addis-Slonaker DNA during the first 10 days after insemination, a total dose of 174 mg DNA; she produced a litter of 13 rats, all of which were jaundiced. Three of these rats survived to the age of 6 weeks, at which time their serum bilirubin levels varied between 6 and 7 mg/100 ml, levels typical of untreated jaundiced rats in the colony.

*Discussion.* We have not been able to produce somatic or genetic transformations with DNA in the rat. It is possible that the biological activity of the DNA was destroyed during the isolation process. This seems unlikely in view of the mild chemical procedure used. The amount of DNA injected into these rats, in relation to body weight, was approximately 10 times as great as that given to ducks by Benoit *et al.* (personal communication). A likely explanation for the failure is that DNA when injected into a mammal fails to reach the interior of cells, where its action might take place. The injected DNA would have to cross several cell membranes, as well as to be carried in the blood stream to sites distant from point of injection. During this process it would be subject to destruction by naturally occurring deoxyribonuclease. There is a vast difference here from the situation where single bacterial cells are bathed in a medium containing foreign DNA, and there is only one cell membrane to cross. The possibility also exists that many cells of a higher organism must be transformed genetically to produce an observable somatic effect. In bacterial transformations, only a fraction varying between 0.01% and 15% of the cells present are permanently modified by foreign DNA. If only 1 in 10,000 skin cells were changed so as to produce melanin in an albino rat treated with DNA derived from a pigmented rat, it might not be possible to detect such a minute change by simple observation. In the case of rats that lack glucuronyl transferase in the liver, it was hoped that, if even a small fraction of the hepatic cells could be changed so as to produce the missing enzyme, bilirubin could be cleared from the serum to some extent. Since this failed to occur, it is probable that the injected normal DNA had no effect in altering the abnormal liver cells.

*Summary.* DNA derived from black and white rats, on injection into newborn albino rats, failed to produce pigmentary changes in the hair and the eyes. Similarly, DNA derived from normal rats failed to correct a genetically determined absence of glucuronyl transferase in newborn jaundiced rats.

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## Influence of 6-Deoxy-6-Fluoroglucose on Glucose Utilization in Rat Epididymal Adipose Tissue and Rat Diaphragm.* (24477)

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The compound 2-deoxyglucose (2-DOG) has been shown to inhibit glucose metabolism as early as the hexokinase step(1). However, one difficulty which has been experienced in studies with this compound is a result of a lack of specificity in its blocking action. Thus it is known that 2-DOG blocks glucose metabolism at the isomerase step(2) as well as the hexokinase step and other sites of action of this drug remain as distinct possibilities (2,3). 6-Deoxy-6-fluoroglucose (6-FG) is a glucose analogue which may circumvent the difficulty of multiplicity of action. Because of the absence of a hydroxyl group in the 6 position, the sugar obviously cannot be phosphorylated by hexokinase. Thus it would appear that if 6-FG does competitively inhibit glucose metabolism in mammalian tissues it should do so prior to or at the hexokinase step. Studies of 6-FG action in rat kidney tissues have indicated that this compound is indeed a competitive inhibitor of glucose utilization (4). Moreover, it appears that 6-FG does inhibit at or prior to the hexokinase step in rat kidney slices. A surprising feature of the study with rat kidney tissue has been the anomalous effect of an increased concentration of 6-FG on glucose oxidation. At low 6-FG concentrations a true competitive response to increasing concentrations of the fluoro derivative is observed. At high 6-FG concentrations, however, a plateau is observed in the curve of inhibition of glucose oxidation over a wide range of 6-FG values. These data have been interpreted to suggest the presence of an alternate pathway of glucose metabolism before the hexokinase step. The object of the studies reported here is to determine whether the inhibitory pattern observed with rat kidney tissues is applicable to other mammalian tissues.

*Material and methods.* The procedures employed in the preparation and incubation of rat adipose tissues and isolated rat diaphragms have been previously described(5,6). When used as substrates concentrations of glucose and acetate were maintained at 0.01M in the incubation medium. 2-DOG was obtained from Nutritional Biochemicals Corp., Cleveland, O. 6-FG was prepared according to the procedure of Helferich *et al.*(7,8) as revised by Blakley(9).

*Results.* The data of Table I show that fluoride ion is an effective inhibitor of oxidation of glucose and acetate in rat epididymal

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TABLE I. Comparison of Effects of 6-Deoxy-6-Fluoroglucose and Fluoride Ion on Oxidation of C14-Labeled Acetate and Glucose in Rat Adipose Tissue.

| Inhibitor | Substrate | Inhibition, % |
|---|---|---|
| 6-FG (.03 M) | U. L. glucose | 51 |
| | Acetate-1-$C^{14}$ | −6 |
| $F^-$ ( " ) | U. L. glucose | 90 |
| | Acetate-1-$C^{14}$ | 96 |
| $F^-$ (.003 M) | U. L. glucose | 64 |
| | Acetate-1-$C^{14}$ | 42 |

adipose tissue. On the other hand, 0.03M 6-FG is effective only as an inhibitor of glucose oxidation.

The influence of 6-FG and 2-DOG concentrations on oxidation of glucose to $CO_2$ in the isolated rat diaphragm is shown in Fig. 1. At low concentrations, 6-FG is as effective an inhibitor of glucose oxidation as 2-DOG. However, with increasing concentrations of these glucose analogues, 2-DOG is seen to exhibit a more potent inhibition of $C^{14}O_2$ formation.

A similar pattern is seen to exist in rat adipose tissue (Fig. 2) with the exception that the differential between the two curves is not as extensive at high inhibitor concentrations.

Comparison of the relative effectiveness of 6-FG as an inhibitor of glucose utilization in rat kidney tissue(4), rat adipose tissue, and isolated rat diaphragm is given in Fig. 3. The effectiveness of 6-FG as an inhibitor is seen to increase in the series: kidney, diaphragm, adipose.

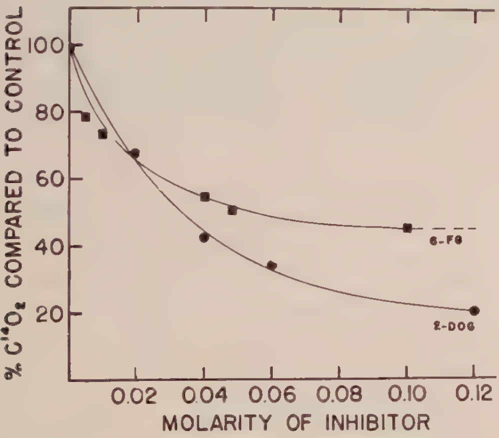

FIG. 1. Effect of 6-FG and 2-DOG on glucose oxidation to $CO_2$ in isolated rat diaphragm. Each point represents avg of 3 separate determinations.

*Discussion.* The inhibitory action of fluoride ion on glycolysis scheme and the Krebs' cycle is a well established phenomenon. Thus the possibility arises that the inhibition observed with 6-FG is not an effect of 6-FG *per se* but rather an effect of fluoride ion hydrolyzed from this glucose analogue during tissue incubation. This possibility appears to be refuted by the data of Table I. A further observation consistent with the conclusion that fluoride ion is not responsible for the 6-FG effect is that 6-FG has been shown to behave kinetically as a competitive inhibitor of glucose utilization(4). Fluoride ion on the other hand is known to be a non-competitive inhibitor of glycolysis.

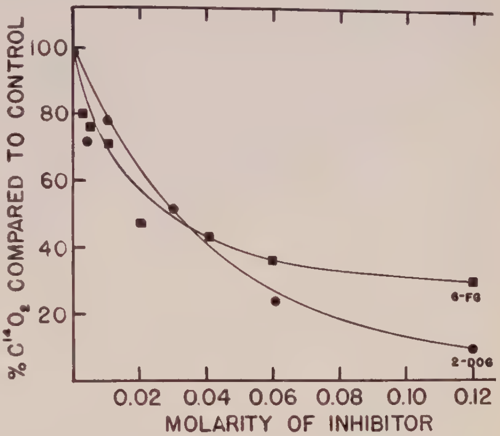

FIG. 2. Effect of 6-FG and 2-DOG on glucose oxidation to $CO_2$ in rat epididymal adipose tissue. Each point represents avg of 3 separate determinations.

Earlier studies with rat kidney slices and sugars variously labeled with $C^{14}$ have indicated that the site of 6-FG action occurs prior to formation of glucose-6-phosphate(4). Moreover, it has been suggested that an unknown pathway leading to glucose oxidation exists in kidney tissues prior to the hexokinase step. This conclusion was reached on the basis of the observation that at high 6-FG concentrations the curve of inhibition of glucose oxidation to $CO_2$ approaches a maximum at approximately 35 to 45% inhibition, indicating that some non-6-FG sensitive pathway is permitting glucose oxidation while the 6-FG sensitive pathway is almost completely inhibited.

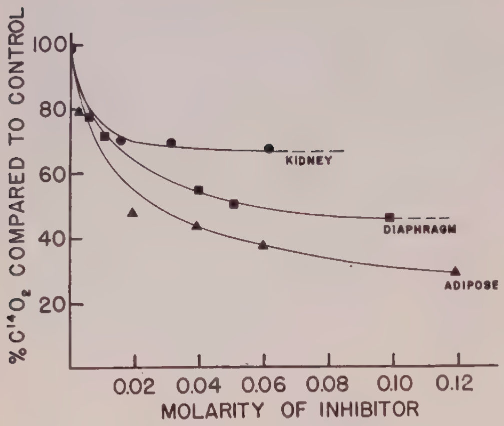

FIG. 3. Comparison of effect of 6-FG on rat kidney, rat diaphragm, and rat epididymal adipose tissue.

The pattern observed in kidney tissue appears to be qualitatively repeated in rat diaphragm (Fig. 1) and rat epididymal adipose tissue (Fig. 2).

Comparison of the response of all 3 tissues to 6-FG concentration (Fig. 3) indicates that the inhibited pathway of glucose oxidation contributes to an increasingly greater extent to total formation of $CO_2$ in the series: kidney $<$ diaphragm $<$ adipose.

The demonstration that 6-FG inhibits glucose oxidation in such diverse specialized tissues as kidney, diaphragm, and adipose as well as in lower organisms such as yeast preparations(10) suggests that the process interfered with by 6-FG is a universal metabolic pathway. Work to determine the exact site of action of this analogue is currently underway.

*Summary.* The effect of 6-deoxy-6-fluoroglucose (6-FG) on oxidation of glucose has been examined *in vitro* in rat epididymal adipose and rat diaphragm tissue. It was found that 6-FG at all concentrations studied was an effective inhibitor of the oxidation of glucose. However, at high 6-FG concentrations, inhibition curve of glucose oxidation approaches a maximum at approximately 75% and 50% for adipose and diaphragm tissue respectively. The inhibition curve obtained with 2-deoxyglucose, which was used for comparison purposes, appeared to asymptotically approach 100% inhibition of glucose oxidation in these tissues. These results have been interpreted to mean that 2 or more pathways previous to the hexokinase reaction appear to exist for oxidation of glucose in these tissues, and that only one of these pathways is inhibited by 6-FG.

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# Sulfur Amino Acid Requirement for Lactation in the Rat.*† (24478)

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The classic studies of Rose(2) on amino acid requirements for post-weaning growth and nitrogen balance in rats have not been extended to the study of amino acid requirements for either pregnancy or for lactation. Numerous reports have been made, however, on the beneficial effects of cystine or methionine supplementation for lactation in rats maintained on diets containing protein sources known to be low or lacking in sulfur amino acids, *e.g.* casein(3,4), alfalfa proteins(5,6), peanut meal(7), and yeast protein(8). In fact, cystine and methionine have been proposed as specific dietary lactogogues(3). In the present study purified diets varying in casein level and in cystine supplementation have been used to determine an approximate value of total sulfur amino acid requirement for optimal lactation in the Long-Evans rat.

*Methods.* The short-term lactation test of 3 weeks is the same as used in previous studies(1,9,10,11). Normal female rats of Long-Evans strain, together with their litters, were placed on experimental diets at parturition. All groups were balanced in regard to average age and body weight of mother at parturition. The litters were limited to 6 young, preferably 3 males and 3 females. Mother and young were weighed every 5 days and the young were weaned on 21st day following parturition. Dietary casein levels of 12, 18, 24, 30, 36 and 48% were used, with sucrose and casein being interchanged isocalorically in the basal purified diet.‡ L-cystine (0.5%) was added to the 12, 18, 24, and 30% casein diets. In addition, 0.2% L-cystine and 0.2% DL-methionine were tested separately with the 24% casein diet. Ten to 12 litters were used for each experimental group and all groups receiving the standard level of 24% casein were repeated. Control litters were maintained on stock diet of natural foodstuffs which contained approximately 24.3% protein (N x 6.25) or the protein equivalent to that furnished by 28% casein purified diet. All diets were given *ad lib.* and food intake was measured every 5 days. Criteria used for lactation performance in this short-term lactation test were those used previously by Nelson & Evans(9): (1) average weaning weight of young, and (2) average weight change of lactating mother during the 21-day period.

*Results.* Table I demonstrates improvement in lactation performance resulting from cystine supplementation of casein-containing purified diets. Addition of 0.5% L-cystine to 12, 18 and 24% casein diets resulted in significantly heavier weaning weights of young and significant improvement in maternal weight change. Cystine supplementation of the 30% casein diet improved weaning weights of young significantly with less beneficial effects on maternal weight. As judged by

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* A preliminary report was presented before Am. Chem. Soc., Portland, Ore., Sept. 1948(1).

† Aided by grants from Board of Research and College of Agriculture, Univ. of California and the Roche Anniversary Fn. We are greatly indebted to Dr. Randolph Major of Merck, Rahway, N. J. for generous supplies of L-cystine, crystalline B vitamins, and 2-methyl-1, 4-naphthoquinone; to Dr. E. L. Severinghaus of Hoffmann-LaRoche, Nutley, N. J. for crystalline *d*-biotin; and to Dr. T. H. Jukes, Lederle Labs., Pearl River, N. Y. for synthetic pteroylglutamic acid.

‡ The basal purified diet contained 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated vegetable oil and 4% salts No. 4(12). Crystalline vitamins/kilo diet were: 300 μg *d*-biotin, 5.5 mg pteroylglutamic acid, 5 mg 2-methyl-1,4 naphthoquinone, 5 mg thiamine HCl, 5 mg pyridoxine HCl, 10 mg riboflavin, 10 mg p-aminobenzoic acid, 20 mg niacin or niacinamide, 50 mg *d*-calcium pantothenate, 400 mg inositol and 1 g choline chloride. One cc of fat-soluble vitamin mixture containing 6 mg *dl*-alpha-tocopherol, 115 chick units vit. D, 800 U.S.P. units vit. A and 650 mg corn oil (Mazola) was given weekly to each litter. Vit. $B_{12}$ was not included in the vitamin mixture for this study. However, succeeding studies have shown that addition of 25-50 μg crystalline vit. $B_{12}$/kilo of the 24% casein diet did not improve lactation performance either in presence or absence of cystine supplementation.

TABLE I. Effect of Supplementary Cystine on Lactation in the Long-Evans Rat. Three-week test—litters limited to 6 young.

| Dietary casein level, % | Cystine suppl., % | No. of litters | No. of young | Avg wt young day 21,* g | Maternal wt change during lactation, g | Avg daily food intake, g | Dietary sulfur amino acids Methionine + cystine = total SAA† |
|---|---|---|---|---|---|---|---|
| | | | Casein-containing purified diets | | | | |
| 12 | .0 | 11 | 66 | 31 ± 2 | −43 ± 8 | 23 ± 1 | .33 + .04 = .37 |
| | .5 | 10 | 60 | 41 ± 2 | −14 ± 3 | 29 ± 1 | .33 + .54 = .87 |
| 18 | .0 | 13 | 78 | 41 ± 1 | −24 ± 2 | 27 ± 1 | .50 + .06 = .56 |
| | .5 | 12 | 72 | 54 ± 1 | 0 ± 3 | 34 ± 1 | .50 + .56 = 1.06 |
| 24 | .0 | 34 | 204 | 49 ± 1 | − 1 ± 2 | 31 ± 1 | .66 + .08 = .74 |
| | .2‡ | 23 | 138 | 54 ± 1 | + 2 ± 3 | 33 ± 1 | .66 + .28 = .94<br>.86 + .08 = .94 |
| | .5 | 36 | 216 | 54 ± 1 | +17 ± 2 | 35 ± 1 | .66 + .58 = 1.24 |
| 30 | .0 | 12 | 72 | 50 ± 1 | − 2 ± 3 | 32 ± 1 | .83 + .10 = .93 |
| | .5 | 12 | 72 | 54 ± 1 | + 9 ± 4 | 34 ± 1 | .83 + .60 = 1.43 |
| 36 | .0 | 12 | 72 | 52 ± 1 | +19 ± 4 | 33 ± 1 | .99 + .12 = 1.11 |
| 48 | .0 | 12 | 72 | 51 ± 1 | +18 ± 5 | 34 ± 1 | 1.32 + .16 = 1.48 |
| | | | Stock Diet I + lettuce *ad lib.* | | | | |
| 28§ | | 102 | 612 | 49 ± 1 | +14 ± 2 | 35 ± 1 | |

* This column gives avg weaning wt of young as calculated from litter avg weights, each litter corrected to the 3:3 sex ratio by means of mid-sex correction factor (CF). All columns give the mean together with stand. error of mean.

† Calculated from standard tables(13) and expressed in proportion to 16% nitrogen.

‡ 0.2% L-cystine was used for 12 litters and 0.2% DL-methionine for 11 litters. The data have been combined as there was no significant difference between the 2 groups.

§ The protein content of stock diet is equivalent to that furnished by a 28% casein-containing purified diet.

both young and mother, optimal casein level in the presence of additional cystine was between 18% and 24%, whereas in the absence of cystine supplementation optimal casein level was probably close to 36%.

The data suggest that slightly more than 1% of total dietary sulfur amino acids (SAA) was necessary for optimal lactation of Long-Evans rats, *i.e.* 1.11 to 1.24% SAA. This SAA requirement for optimal lactation is considerably higher than that needed for post-weaning growth of rats as determined by Wretlind and Rose(14) *i.e.* 0.5-0.6% SAA furnished by 0.3-0.4% methionine and 0.2% cystine. There are several possible explanations for this difference, *e.g.* (1) higher SAA requirements for lactation than for growth, (2) strain differences in SAA requirement and (3) loss or non-availability of methionine or cystine resulting from process of alcohol-extraction.§

The purified diets containing 18-30% casein and supplemented with cystine resulted in young with weaning weights superior to those produced previously by similar diets supplemented with 8 B vitamins and a liver eluate powder(10). Average weaning weights of 54 g on these cystine-supplemented diets closely approached the optimal weaning weights of the Long-Evans rat reported by Dunn *et al.*(15) *i.e.* 54 g for females and 56 g for males.

*Summary.* A short-term lactation test has been used to study the effects of cystine supplementation for purified diets containing casein as sole protein source. Supplementation of 12, 18, and 24% casein diets with 0.5% L-cystine markedly improved lactation performance as judged by weaning weights of young and mother. Cystine supplementation of 30% casein diet improved weaning weights of young significantly with less beneficial effects on maternal weight. The data indicate that total sulfur amino acid requirement for optimal lactation in the Long-Evans rat may be placed at slightly more than 1% of the diet and that cystine may furnish at least half of this requirement.

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§ Microbiological assays for methionine with *L. fermenti*$^{36}$ showed an apparent loss of methionine in all alcohol-extracted caseins tested. Methionine content for casein used was only 2.65% in comparison with the value of 3.3% given in standard tables(13) and with that of 3.2% for casein prepared by isoelectric precipitation and analyzed by the same microbiological assay at the same time as the alcohol-extracted caseins. These assays were carried out by Dr. S. Shankman of Shankman Laboratories, Los Angeles, Calif. Alcohol-extraction had no apparent effect on cystine content of casein preparations when cystine was assayed with *L. mesenteroides*.

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## Relationship between Pituitary Growth Hormone Content and Age in Rats.* (24479)

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The question of whether or not the amount of pituitary growth hormone varies with age has been asked by several laboratories using beef(1), swine(2), and fetal and infant rat pituitaries(3) in the tibia test assay. To date, there is no consistent opinion as to the relationship between growth rate and amount of growth hormone in the pituitary. To determine whether growth hormone potency of the female rat pituitary varies as the animal ages from 10 to 630 days of age, the following methods were employed:

*Methods.* Female rats descended from the Sprague-Dawley and Wistar strains were weighed and sacrificed with ether at 10, 42, 56, 70, 168, 308, 376, and 630 days of age. At necropsy, the pituitaries were removed, weighed, and placed in absolute acetone, dried and stored in a vacuum desiccator for use in the tibia test assay for growth hormone. The growth hormone potency of the pituitaries was determined according to the procedure of Evans *et al.*(4) using the parallel line method. For each assay, the whole dried pituitaries were weighed, triturated with saline and injected at 2 dose levels; and 3 groups of hypophysectomized rats received graded doses of a purified beef growth hormone preparation (Armour and Co.). Pituitaries from 2 age groups (42 and 70-day-old females) were assayed twice to test the accuracy of the procedure.

*Results.* Table I shows epiphyseal widths and statistical evaluation of growth hormone potency of the donor pituitaries. Table II is a compilation of data, using body and pituitary weights of the donor rats, and assayed growth hormone content of the pituitaries, expressed in terms of μg of growth hormone/mg pituitary, or per g rat.

Within the limits of the assay procedure,

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* Aided by research grant from Nat. Inst. Health, U.S.P.H.S., and support from Am. Cancer Soc., Mass. Division.

TABLE I. Pituitary Growth Hormone Potencies of Female Rats 10 to 630 Days of Age.

| Source of pituitary preparation | No. of hypox. rats | Dose, μg/day | Epiphyseal width, μ | R value, μg/mg pituitary | Slope (b) | Stand. dev. (s) | Lambda (λ) |
|---|---|---|---|---|---|---|---|
| 10-day-old rat | 5 | 250 | 172 ± 9.28 | 79.8 | 188.48 | 20.76 | .152 |
| *Idem* | 2 | 500 | 228 ± 14.68 | | | | |
| 630-day-old rat | 5 | 250 | 173 ± 9.28 | 102.0 | 375.87 | | |
| *Idem* | 3 | 500 | 257 ± 12.00 | | | | |
| Beef growth hormone | ” | 10 | 154 ± 12.00 | | | | |
| *Idem* | 5 | 25 | 191 ± 9.28 | | 96.58 | | |
| ” | ” | 50 | 221 ± ” | | | | |
| Hypophysectomized controls | ” | | 145 ± ” | | | | |
| 42-day-old rat | 6 | 250 | 234 ± 7.84 | 127 | 93.02 | 19.19 | .217 |
| *Idem* | ” | 500 | 262 ± ” | | | | |
| 56-day-old rat | ” | 250 | 203 ± ” | 94 | 141.20 | | |
| *Idem* | ” | 500 | 246 ± ” | | | | |
| 70-day-old rat | ” | 250 | 214 ± ” | 116 | 177.74 | | |
| *Idem* | ” | 500 | 267 ± ” | | | | |
| Beef growth hormone | ” | 10 | 176 ± 8.58 | | | | |
| *Idem* | 5 | 25 | 197 ± 7.84 | | 61.60 | | |
| ” | 6 | 50 | 219 ± ” | | | | |
| Hypophysectomized controls | ” | | 145 ± ” | | | | |
| 42-day-old rat | 6 | 250 | 230 ± 9.88 | 165.0 | 129.57 | 24.14 | .220 |
| *Idem* | ” | 500 | 269 ± ” | | | | |
| 308-day-old rat | ” | 250 | 182 ± ” | 77.6 | 207.09 | | |
| *Idem* | ” | 500 | 245 ± ” | | | | |
| Beef growth hormone | 5 | 10 | 179 ± 10.80 | | | | |
| *Idem* | 6 | 25 | 195 ± 9.88 | | 85.92 | | |
| ” | ” | 50 | 239 ± ” | | | | |
| Hypophysectomized controls | 5 | | 142 ± 10.80 | | | | |
| 70-day-old rat | 5 | 500 | 236 ± 11.58 | 99.9 | 75.30 | 25.90 | .216 |
| *Idem* | 6 | 1000 | 258 ± 10.58 | | | | |
| 378-day-old rat | ” | 500 | 240 ± ” | 96.1 | 58.14 | | |
| *Idem* | ” | 1000 | 257 ± ” | | | | |
| Beef growth hormone | 5 | 25 | 224 ± 11.58 | | | | |
| *Idem* | 6 | 50 | 257 ± 10.58 | | 146.46 | | |
| ” | ” | 100 | 313 ± ” | | | | |
| Hypophysectomized controls | ” | | 142 ± ” | | | | |
| 168-day-old rat | 4 | 250 | 169 ± 12.40 | 44.6 | 88.61 | 24.80 | .695 |
| *Idem* | 5 | 500 | 195 ± 11.09 | | | | |
| Beef growth hormone | 4 | 10 | 181 ± 12.40 | | | | |
| *Idem* | 3 | 25 | 186 ± 14.32 | | | | |
| ” | ” | 50 | 197 ± ” | | 23.18 | | |
| Hypophysectomized controls | 2 | | 147 ± 17.54 | | | | |

the values obtained for amount of growth hormone in the female rat pituitary indicate that growth hormone may vary in rats of the same age as well as between age groups. Since body and pituitary weights increase with time, while growth hormone potency remains relatively static, there is no consistent trend of increasing or decreasing activity per milligram pituitary or per gram rat.

*Summary.* Pituitary growth hormone content of female rats from several age groups was assayed in the tibia test; and it was found that the amount of growth hormone, expressed in either μg/mg pituitary or in micrograms growth hormone/g rat, does not follow an ascending or descending course but fluctuates within a relatively small range as the rat ages from 10 to 630 days of age.

TABLE II. Micrograms Growth Hormone per Milligram Pituitary and per Gram Rat.

| Age, days | No. rats | A<br>μg growth hormone/mg pituitary | B<br>mg pituitary | C<br>Body wt, g | $\frac{A \times B}{C}$<br>μg growth hormone/g rat |
|---|---|---|---|---|---|
| 10 | 20 | 79.8 | 1.39 | 24.10 | 4.60 |
| 42 | 19 | 127.0 | 4.78 | 99.00 | 6.13 |
| " | " | 165.0 | 4.78 | 99.00 | 7.97 |
| 56 | 15 | 94.0 | 5.96 | 119.66 | 4.68 |
| 70 | 20 | 99.9 | 7.79 | 143.00 | 5.44 |
| " | " | 116.0 | 7.79 | 143.00 | 6.32 |
| 168 | 16 | 44.6 | 20.56 | 326.81 | 2.81 |
| 308 | 15 | 77.6 | 19.38 | 351.60 | 4.28 |
| 378 | 33 | 96.1 | 20.28 | 358.10 | 5.44 |
| 630 | 15 | 102.0 | 25.06 | 427.80 | 5.97 |

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## Use of Glass Electrode for Measuring Sodium in Biological Systems.*†

(24480)

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In seeking to trace the meaning of the relationship between sodium and blood pressure we have been hampered by difficulties inherent in estimation of small quantities of this cation. While it is true that sodium movements regularly accompany changes in blood pressure(1) the magnitude of the shifts lies just on the borderline of what can be determined without resort to statistical analysis of small differences. Similarly, while we have shown that pitressin has extrarenal actions on sodium, we were forced to demonstrate these with relatively large doses in order to produce effects measurable by existing methods (2). A further major problem in these studies has been the fact that the time course of ion shifts can only be crudely estimated by blood sampling procedures. Recently, Eisenman, Rudin and Casby(3) reported on preparation of cation sensitive glasses and indicated their potential usefulness as electrodes for biological studies. The important implications of this work led us to explore, with the cooperation of Dr. Eisenman, the technical problem of adapting the electrodes for use in biological systems. The present report concerns only the sodium electrode.

*Methods. Amplifying and recording circuitry.* The basic electronic problem involves suitable high gain amplification from a high impedance source. Since a change of 1 meq/l concentration from a base of 150 meq/l should ideally yield a potential change of approximately 160 microvolts it is evident that the drift and noise characteristics of the instrumentation are the limiting factors in sensitivity. The Cary vibrating reed electrometer is a very satisfactory basic unit to meet these requirements, having a limiting sensitivity of about 20 microvolts and a very low drift rate.

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* This work was carried out with the aid of a grant from the Muscular Dystrophy Assn. of Canada.

† We are grateful to Mr. John Lees for his skilled help in manipulating the glass.

The output of the electrometer is read directly on its panel meter. Additionally, it is fed into a direct writing oscillograph. A calibrated bucking potential is applied between electrode and amplifier so that net input potential can be limited to the most sensitive ranges of the instrument. In practice, we use a Grass Model 5 Polygraph as recording unit, receiving the electrometer output through a low-level DC preamplifier. The balance voltage circuit of the latter unit is externalized to serve conveniently as the source of the bucking potential. *Glass electrodes.* NAS$_{11-18}$ glass, generously supplied by Eisenman, Rudin and Casby, was used throughout for fabrication of electrodes. In all cases, a standard calomel reference electrode was used. *A. Haber bulb electrode.* Measurements were made using Haber type bulbs filled with 0.1 M NaCl and containing a central Ag/AgCl lead according to the procedure of Eisenman *et al.* These proved suitable for estimation of large differences of Na concentration where relatively low amplifications are required. Fine measurements were not possible with this arrangement since solutions cannot be changed without considerable electrical interference. The bulb was then incorporated into a cannula into which solutions were fed from burettes at a distance. The cannula itself was shielded by a grounded box. This arrangement permitted work at high ranges of the amplifier and several versions of the assembly were tested. We were able to confirm the fact that the sodium sensitive glass responds only to Na$^+$ within the usual biological ranges for H$^+$ and K$^+$ background. We further observed that the response remains linear at high amplifications over small changes in Na concentration. We were, however, unable to provide any long-lived insulation for the stems of the bulbs despite trials with a wide variety of materials. This meant that we were continually plagued with fabrication of new electrodes until one of us (J. A. M. H.) simplified the problem by constructing the cannula itself of NAS$_{11-18}$ glass. *B. Glass-to-silver electrode.* A glass-to-silver electrode in tubular form is shown in Fig. 1. NAS$_{11-18}$ glass is blown into a thin-walled fusiform bubble which is silvered on the outside by the Rochelle method. The silver in turn in heavily copper plated with a lead wire incorporated directly into the copper deposit. Polyethylene tubing is attached to both ends, the outside of the assembly is heavily coated with a non-hardening bakelite for insulation, then housed in a plastic box for protection. The lead wire is connected to a screw terminal since hot solder connections cannot be used. These glass-to-silver electrodes have proved rugged and long-lasting. For unknown reasons they have not yielded the ideal 58 mv slope but have settled at approximately 48 mv with remarkable day to day stability. The results described below have all been obtained with the tubular glass-to-silver electrode, measuring 0.5 x 2.0 cm, although the size does not appear to be critical.

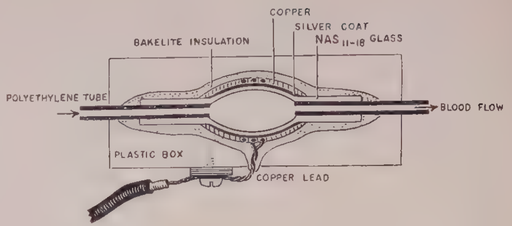

FIG. 1. Diagrammatic section through a tubular type of glass-to-silver sodium electrode. Measurements are approximately 0.5 × 2.0 cm.

*Results. In vitro.* The potentials developed by the electrode in static and flowing unbuffered systems differ by a constant (Fig. 2) which is itself a function of the rate of flow. The constant for a flow rate of 45 ml/min. compared with zero flow is almost 1 mv. Although this is a rather extreme situation, it is evident that rate of flow must be evaluated in any attempt to measure small changes in Na concentration.

Rate of change of flow, or acceleration, is another factor which may cloud the interpretation of electrode potentials in unbuffered systems. This factor can be demonstrated easily in start-stop experiments where an overshoot occurs on starting the flow through the tube; height of the overshoot can be reduced almost to the vanishing point by reducing the rate at which flow starts.

Changes in flow rate are less important with buffered solutions. Thus, with Na phosphate at pH 7.3, changing the flow rate from zero to 4, 6, 8, 10, 15 or 24 ml/min. gave a po-

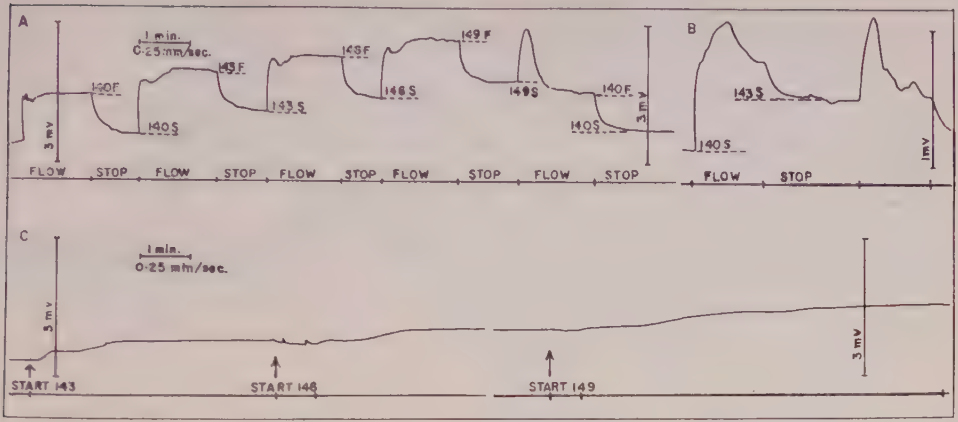

FIG. 2. Effect of altering Na (acetate) concentration in steps of 3 meq/l, between 140 and 149 meq/l, on sodium electrode potential. *A*. Potentials under static (S) and flowing (F) conditions. Flow rate is standard at 45 ml/min. *B*. Potential difference for a 3 meq/l step in concentration, amplified 3-fold in comparison with tracing in *A*. *C*. Electrode potentials recorded for 3 meq/l steps under continuous flow conditions. The increment for each step is the same as in *A*.

tential change of 50 to 100 microvolts while no change occurred once the system was flowing when the rate was changed from 4 to 18 ml min.

Within the limitations noted above, fractional changes in Na concentration can be measured reproducibly. Thus, without serious attempt to reduce noise, changes of 50 microvolts, *i.e.*, of 0.3 meq/l out of 150 meq/l of Na can readily be distinguished. This sensitivity is considerably better than the 1% limit of the most careful flame determination and it is evidently possible to reduce this towards the 20 microvolt sensitivity limit of the instrument. A big advantage of the electrode is its ability to follow cyclic changes reproducibly without noticeable lag. Drift is ordinarily negligible for short term measurements and is easily compensated in longer procedures. The electrode can be accidentally polarized by the potential from the bucking source and this must be carefully avoided in switching. A stabilization period of at least 15 minutes is required after any interruption of the circuit.

*In vivo*. For this study, adult female rabbits, anesthetized with barbitone-urethane and fully heparinized to eliminate the possibility of clotting in the cannula, were used. The aim was to determine whether the electrode was able to follow cyclic changes in Na concentration in blood. The tube electrode was implanted as a by pass into the femoral artery. Blood pressure was recorded simultaneously from the opposite femoral artery using a Statham P23AA transducer. Infusions were given through polyethylene tubing implanted in the femoral vein. The calomel reference electrode was firmly anchored into the wound ooze.

The ability of the electrode to follow cyclic changes of Na concentration in whole blood was clearly demonstrated by infusion of measured amounts of Na as acetate or phosphate. Electrode response is a direct function of the amount of Na infused (Fig. 3). With each infusion there was a brief latent period, apparently corresponding to circulation time, followed by a rapid increase in electrode potential reflecting the initial small volume of distribution of the infused Na, and a gradual subsidence again as the Na became uniformly distributed. With each injection base line Na concentration rose in proportion to amount administered. Thus, the electrode is measuring Na concentration or, more accurately, activity.

In previous studies we have shown that Na concentration in the blood is related to the blood pressure. The electrode should follow these changes more faithfully than the crude sampling method originally used to demonstrate this relation. In Fig. 4 the typical coordinated response of blood pressure and Na level to acute injection of norepinephrine is shown. Not only are the changes in Na concentration grossly similar to those shown chemically but they are here followed with a detail not previously possible.

*Discussion.* The $NAS_{11\text{-}18}$ glass developed by Eisenman, Rudin and Casby responds only to sodium at a pH greater than 4 and in presence of a $K^+$ concentration less than 30 meq/l. This means that if high amplification is possible, this glass can be used for specific measurement of Na in ordinary biological systems. The present report has demonstrated that such amplification is feasible and that glass-to-silver electrodes are particularly suitable for this purpose. We have used the electrode in the form of a tube or cannula but evidently other arrangements are equally possible. Thus, the tube could be closed at one end and silvered either on the inside to form an immersion tip or on the outside to form a containing tube. Enlargement of the cannula to a bath in which muscle strips might be studied, or its contraction to microelectrodes should be equally feasible.

Thus far, we have been concerned only with ability of the electrode to measure relative changes in sodium activity. Additional problems require solution if the electrode is to be used for measurement of absolute quantities of sodium involved in such dynamic changes. Such studies are underway. Further, the usefulness and range of the glass-to-silver sodium electrode would be enhanced if simultaneous measurements of potassium could also be made. Preliminary studies indicate that the $K^+$ electrode may also be applicable to biological work.

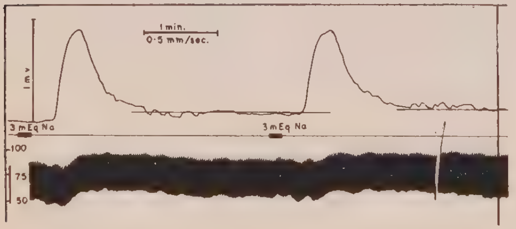

FIG. 3. Blood pressure (lower trace) and sodium electrode potential recorded simultaneously from femoral artery of a female rabbit of 3.5 kg. Sodium acetate (3 meq in 0.75 ml) was inj. rapidly as indicated by the bar. (Electrode potential, 48 mv/log unit.)

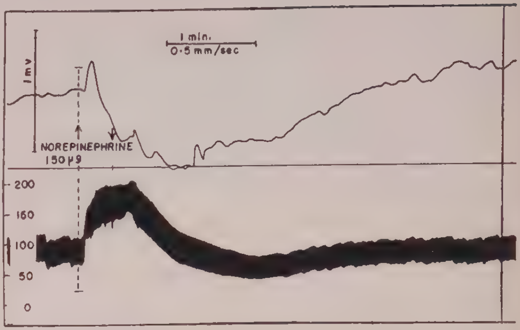

FIG. 4. Blood pressure and sodium electrode potential recorded simultaneously from femoral artery of a female rabbit of 3.5 kg. In the interval indicated by the arrows, 150 µg of norepinephrine (bitartrate) was inj. intrav.

*Summary.* Development of a sodium glass-to-silver electrode for biological use has been described. The instrument is simple and particularly useful for continuous recording of small changes in sodium concentration, both in static and in flowing systems. As such, it should be applicable to a wide variety of studies.

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# Comparative Effectiveness of Fresh and Lyophilized Platelets in Controlling Irradiation Hemorrhage in the Rat.* (24481)

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It has been established that thrombocytopenia is the major cause of hemorrhage in animals exposed to uniform whole body irradiation(1). Repetitive transfusions of fresh platelets, to maintain reasonable platelet levels, prevent hemorrhage. Bleeding that has commenced can be quantitatively suppressed by a single fresh platelet transfusion (2). The storage life of platelets is measured in hours. Accordingly, considerable efforts have been exerted to preserve platelets by freezing(3), storage in media(4), or by lyophilization(5). Platelets preserved by the preceding 3 methods have been reported of value in controlling bleeding in leukemic children(5). However, the methods of assaying effectiveness of platelet transfusions in patients are not sufficiently quantitative. Since radiation injury may occur accidentally in the atomic industry and would be seen on a large scale in nuclear warfare, and intermittently after chemotherapy of cancer, it appeared essential to evaluate the effectiveness of preserved platelets in controlled radiation injury by quantitative methods. This report deals with the comparative effectiveness of fresh and lyophilized platelets in controlling hemorrhage in fatally irradiated rats.

*Methods and materials.* Sprague-Dawley rats were used, females and males approximately 100 g at time of irradiation. Donor rats were cast off female breeders about 300 g in weight. Platelet counts were performed by Brecher-Cronkite phase contrast method(6). Rats were given 600 r total body irradiation by 250 KVP General Electric x-ray machine operating at 30 ma and filtered with 0.5 mm of Cu and 1 mm of Al. Tissue dose rate was 32 r/minute at TSD of 100 cm. Donors were bled from abdominal aorta under sterile conditions into cold siliconed syringes containing 1 ml of cold 1% $Na_2$ EDTA in 0.7% saline. The blood was then placed in cold siliconed tubes and platelets were separated by modification of the differential centrifugation technic of Dillard *et al.*(7). Freshly separated platelets were either transfused within 2 hours or lyophilized precisely according to the method of Klein, *et al.*(5) and stored in sealed ampoules filled with dry nitrogen. In a preliminary experiment to evaluate histologically the effectiveness of platelets in preventing hemorrhage, 18 rats were given 600 r. Three groups of 6 animals each were to have received transfusions of fresh platelets, lyophilized platelets and saline, respectively, on days 6, 8 and 10, with sacrifice to follow on day 11. The amount of fresh platelets transfused was estimated to raise platelet count to between 1 and 2 x $10^6$ platelets/$mm^3$. Equivalent amounts of lyophilized platelet material were given. All animals were transfused on 6th and 8th day. At time of second transfusion on 8th day, one animal receiving lyophilized platelets died within minutes in convulsion. Then one animal from each of the other groups was sacrificed. Similarly on day 10, 2 animals receiving lyophilized platelets died, and 2 from the other groups were sacrificed. The remaining animals were sacrificed on day 11. At sacrifice, tissues were prepared for histological examination. In a second series of experiments, fresh platelets in amounts indicated above, were given on 5th, 7th and 9th post-irradiation days to 5 rats, and lyophilized platelets to 5 rats. All animals receiving transfusions of fresh or lyophilized platelets were sacrificed on day 10. Eight animals were subjected to irradiation only, and 2 were sacrificed on each of post-irradiation days 3, 5, 7 and 10. Four control animals receiving no treatment were sacrificed on day 10. All tissues were prepared for histological examination. In Exp. 2, one animal died

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† Research Collaborator, Johns Hopkins Hospital, Baltimore, Md.

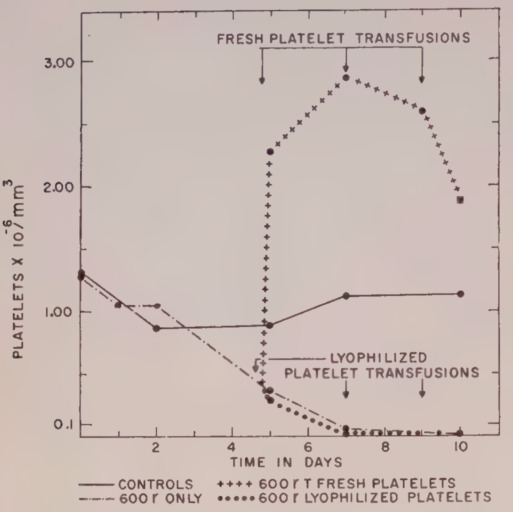

FIG. 1. Effect of transfusions of fresh and lyophilized platelets on platelet count following exposure to total body irradiation in the rat.

shortly after receiving a transfusion of lyophilized platelets on day 9, and was not included in data given above. Several tests were used to assay for presence of bleeding and the effectiveness of transfusions as follows: 1. Gross observation for bleeding, 2. Platelet counts, 3. Study of hematocrit, 4. Observation for and microscopic evidence of bleeding and concentration of red cells in lymph nodes, 5. Susceptibility to bleeding from minor trauma (subcutaneous injection of 0.5 ml of isotonic saline).

*Results.* Fig. 1 presents comparative platelet counts of control non-irradiated rats, control irradiated rats given no transfusions, rats irradiated and given fresh platelet transfusions, and rats irradiated and given lyophilized platelet transfusions. Fresh platelet transfusions raised and maintained the platelet count at more than twice the normal level. There was no detectable difference in platelet count between non-transfused irradiated rats and irradiated rats receiving lyophilized platelet transfusions.

Fig. 2 shows comparative serial hematocrits for all groups of rats. The constancy of the hematocrit in non-irradiated rats demonstrates that blood sampling did not affect the hematocrit. Irradiated non-transfused rats developed the usual anemia expected on the basis of past experience. The decrease in hematocrit of rats receiving platelet transfusions was considerably less rapid than in control irradiated or lyophilized transfused irradiated rats. Rats receiving lyophilized platelets developed a more severe anemia than control irradiated rats.

At autopsy on eighth to eleventh post-irradiation day, diffusely bloody lymph nodes were seen grossly and microscopically in control-irradiated and lyophilized-platelet-transfused groups. Control non-irradiated and fresh platelet transfused rats showed no gross hemorrhage, and only a rare extravascular red cell microscopically in lymph nodes of the platelet transfused group. In all groups, phagocytized red cells were found in the lymph nodes.

At sites of saline injection, ecchymoses were seen in the control-irradiated and lyophilized-transfused groups. No ecchymoses were seen in control non-irradiated or in irradiated fresh platelet transfused groups.

Fresh platelet transfusions were tolerated without visible distress. The lyophilized platelet transfusions regularly produced vessel spasm at site of injection. Frequently, respiratory stress and hyperexcitability followed injection of lyophilized platelets. This passed off promptly in most instances, although several deaths resulted as noted above.

*Discussion.* In the irradiated rat, fresh platelets prevented bleeding completely. Lyophilized platelets in similar amounts were totally ineffective. In the fresh platelet trans-

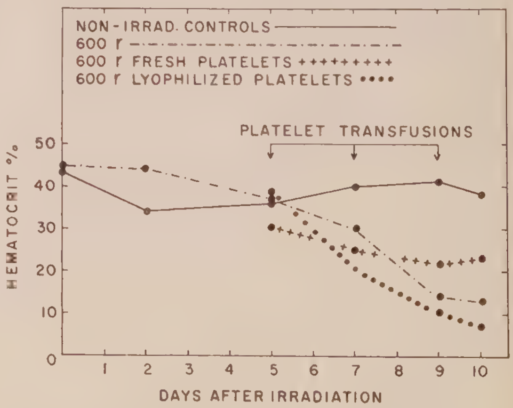

FIG. 2. Effect of transfusions of fresh and lyophilized platelets on hematocrit following exposure to total body irradiation in the rat.

fused group, since there was no hemorrhage, the decrease in hematocrit was primarily due to cessation in new cell production and senescence of red cells present at time of irradiation. If total blood volume remained constant, the red cell mass decrease, as indicated by the hematocrit, would have been about 70% over a 10-day period. Thus a very rough estimate of rat red cell life span by this manner is in excess of 20 days.

It could be argued that larger amounts of lyophilized platelets might be needed to control bleeding. However, the amounts injected were at a level resulting in acute mortality in some animals.

These observations do not apply to frozen platelets and do not prove that lyophilized platelets are of no value in other bleeding states. Since radiation bleeding apparently is due almost exclusively to a simple thrombopenia one can conclude that lyophilized platelets are ineffective in controlling bleeding from uncomplicated thrombocytopenia, whereas fresh platelets are effective in controlling such bleeding.

*Summary.* The efficiency of fresh *vs.* lyophilized platelet transfusions to control radiation induced hemorrhage were tested in rats by gross observation for bleeding, platelet count, hematocrit, microscopical study of lymph node sections, and susceptibility to bleeding from minor trauma. Hemorrhage occurs in irradiated, untreated rats around the fifth post irradiation day increasing with the progress of platelet fall, which reach a minimum around the tenth day. Fresh and lyophilized platelet transfusions were performed on 5th, 7th and 9th day. Whereas fresh platelet transfusions raised the platelet count to 2-3 times the normal and stopped hemorrhage, lyophilized material had no effect on platelet counts which were not different from irradiated controls and did not show any influence on the bleeding tendency. In the lyophilized platelet transfused rats, the hematocrit dropped more than in the irradiated controls.

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## Total Body Water in Growing Domestic Fowl by Antipyrine Dilution Technic. (24482)

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That total body water was nearly a direct proportion of fat-free weight was shown by Light *et al.*(1) to be true for any particular age group but not between ages, in weanling and in grown rats. Annegers(2) studied the relationship among total body water, body fat, and fat-free, dry body weight in normal rats and mice and in starved and dehydrated rats. He showed that body fat did not have an independent effect on total body water when expressed on a fat-free basis. Severe dehydration reduced total body water in adult female rats. Distribution of non-toxic analgesic, antipyrine, in tissues in proportion to their water content was observed by Brodie and Axelrod(3). The volumes of distribution of antipyrine and deuterium oxide were found to agree well in normal subjects. Good agreement has also been obtained when com-

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TABLE I. Total Body Water of Growing White Leghorn Pullets by the Antipyrine Dilution Technic.

| Age, wk | No. of birds | Wt, g | mg antipyrine inj. | Plasma $H_2O$, % | Body water ml | Body water % |
|---|---|---|---|---|---|---|
| 1 | 16 | 68.7 ± 3.8* | 15 | 95.5 ± .3* | 58.5 | 85.2 |
| 2 | 12 | 88.1 ± 9.3 | 15 | 96.3 ± .5 | 60.5 | 68.7 |
| 3 | 17 | 148.5 ± 20.0 | 30 | 96.1 ± .4 | 99.7 | 67.1 |
| 4 | 16 | 221.0 ± 17.9 | 30 | 95.8 ± .2 | 152.3 | 68.9 |
| 6 | 8 | 422.8 ± 24.3 | 60 | 95.8 ± .4 | 250.2 | 59.2 |
| 8 | 16 | 605.8 ± 34.3 | 75 | 95.5 ± .2 | 399.8 | 65.9 |
| 16 | 8 | 1284.0 ± 48.1 | 150 | 95.1 ± .5 | 626.0 | 48.7 |
| 32 | 6 | 1619.3 ±227.5 | 150 | 94.6 ± .3 | 890.7 | 55.0 |

* Stand. dev.

paring volumes of distribution of antipyrine with total body water estimated by desiccation. The validity of antipyrine measurements was further confirmed when compared to calculations from body specific gravity(4, 5). This method has been applied successfully to cattle(6), swine(7) and calves(8). Weiss(9) used the antipyrine dilution technic to estimate total body water in mature chickens. In the work reported here, the antipyrine dilution technic was extended to include stages of growth of chickens from hatching to maturity.

*Materials and methods.* Females of the White Leghorn breed at Cornell University were used. They were fed a commercial ration appropriate to their age and housed in batteries at temperatures within their required zones. Birds of 1, 2, 3, 4, 6, 8, 16 and 32 weeks of age were studied. They were deprived of food for 18-24 hours before determinations were made. An appropriate amount, depending upon size of the bird, of a 15% antipyrine solution was injected intravenously. Blood samples were taken with heparinized syringes at 5, 10, 20 and 30 minutes after injection (in some cases 45 minutes). The problem of obtaining 4 samples was met by injecting a large number of chicks and collecting only one sample from each at a certain time after injection. As the birds grew older, 2 blood samples were taken from one bird. With mature hens all the blood samples could be taken from the same bird. A protein-free plasma filtrate was prepared. Antipyrine was determined essentially as described by Weiss (9). Plasma water was determined by desiccation of plasma to constant weight at 105°C.

*Results.* The results obtained for total body water by the antipyrine dilution technic are presented in Table I. In the 1-week-old chick, total body water was found to be 85.2% of the body weight; this percentage dropped rapidly to begin with and decreased to 55.0 in the mature bird. Plasma water content had a very narrow range from 95.5% in the 1-week-old to 94.6% in the mature hen. The estimation of total body water by desiccation(10) is also shown.

*Discussion.* With the exception of the 1, 6 and 16-week-old birds the direct method and antipyrine method agree fairly closely, although it does appear that antipyrine underestimates total body water. There was considerable variability found in the total body water in the one-week-old chick. This was probably due to differing amounts of yolk present in the birds used. The results obtained for the 32-week-old birds are in agreement with those published by Weiss(9). In conjunction with other published results (1,10) it was found in chickens that the relationship between total body water and fat-free body weight, which holds for any particular age group, does not hold between ages.

*Conclusion.* Total body water was estimated by the antipyrine dilution technic. There was considerable variation between the ages studied. In comparison with the direct method it appeared that in chickens the antipyrine technic underestimates body water, the range being from 85.2% for a one-week-old chick to 55% for the 32-week-old bird.

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## Origin of the *Trans* Fatty Acids in Human Tissue.* (24483)

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We have previously reported(1,2) that *trans* fatty acids were deposited in the tissue of female rats which had been fed *trans* fatty acids and that these acids did not seem to pass through the placenta, as the progeny from these rats did not contain *trans* fatty acids unless they had been allowed to suckle the maternal milk. Since rat and human placentae may differ in ability to transfer nutrients, it is not possible to conclude from these results whether the human baby receives *trans* fatty acids *via* the placenta. In the present study samples of fat from human placental, maternal, fetal, and baby tissue were examined for the presence of *trans* fatty acids to determine whether those in human tissues originate(3) solely from dietary fat.

*Methods.* The placenta was washed free of superficial blood with cold water, the umbilical cord removed; the placenta was cut into small pieces, ground in Waring Blendor in the presence of ethanol, and allowed to remain in contact with solvent for 12 hours. The remaining lipid was removed by successive extractions with acetone, petroleum ether (Skellysolve F) and ethyl ether. Extracts were combined, dried over sodium sulfate and freed from solvent. Since these lipids contained a large amount of unsaponifiable material which interfered with absorption at 10.36 μ in the infrared region, analyses for *trans* fatty acids were carried out on the methyl esters. The combined lipids were saponified with 4% alcoholic potassium hydroxide at room temperature. We found that *trans* isomers are not formed under these conditions. The unsaponifiable material was removed in a separatory funnel by shaking with Skellysolve F, the soaps were acidified with hydrochloric acid, fatty acids removed by extraction with Skellysolve F, free from solvent, and the methyl esters prepared from residue by esterification with cold methanol saturated with hydrogen chloride gas. All extractions were done quantitatively and solvents were removed by use of a Rinco rotating evaporator. Lipids from samples of maternal, baby and fetal tissue were extracted by use of Soxhlet apparatus and after drying, the solvent (acetone followed by Skellysolve F) was removed as described above. The % *trans* double bond was determined at 10.36 μ by use of Beckman IR2A spectrophotometer and the Jackson and Callen baseline method(4). All analyses were done in carbon disulfide solution at 5% concentration. Methyl esters were compared with methyl elaidate standard and all other samples with a trielaidin standard.

*Results.* Methyl esters obtained from placental lipids contained little or no *trans* double bonds. In 3 samples a slight absorption

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* This study was supported by a grant-in-aid from National Livestock and Meat Board, Chicago, Ill.

TABLE I. Percentage of *Trans* Fatty Acids in 6 Samples of Placental and Maternal Fat from Same Individual.

| Placental fat,* % *trans* | Maternal depot fat, % *trans* | Age of mother |
|---|---|---|
| 0 | 6.4 | 16 |
| .5 | 6.8 | 20 |
| 0 | 6.1 | 19 |
| 0 | 1.5 | 26 |
| .5 | 1.8 | 37 |
| 0 | 5.2 | 22 |

* Six other samples of placental fat were collected from which maternal fat had not been available, none contained *trans* fatty acids.

at 10.36 μ was observed, but this was too slight for measurement and is reported as a trace or less than 0.5% (Table I).

In 6 cases of Caesarean section, it was possible to obtain samples of the maternal depot fat in addition to the placenta. Analyses of these samples showed that the depot fat of the mother contained from 1.5-6.8% of *trans* fatty acids, whereas methyl esters of the placental lipids from these mothers contained little or no *trans* double bonds (Table I).

The lipid extracted from 5 samples of fetal liver (5 to 8 months gestation) contained no appreciable amounts of *trans* double bonds. In 4 cases there was no absorption at 10.36 μ and slight absorption was noted in the fifth case but was not measurable. Furthermore, the fat extracted from 2 pooled lots of new born baby tissue[†] representing a total of 22 samples showed little or no *trans* fatty acids were present in this tissue.

While it has been shown that polyunsaturated fatty acids(5) and other lipids do pass the placental barrier, the amounts are apparently small(6). It is therefore improbable that any appreciable amount of *trans* fatty acids would be detected in the placental fat or be passed on to the fetus unless the maternal depot fat and blood contained large quantities of *trans* fatty acids. We have found a maximum of 14% *trans* in human tissue(3) and traces only in the fatty acids of human blood and human milk fat. No correlation was noted between age of the mother and percentage *trans* fatty acid content of her depot fat (Table I).

The results of our study indicate that the *trans* fatty acids present in human tissue apparently arise solely from dietary fat, and as in other non-ruminants(7), they do not normally appear in the tissues unless a source of *trans* fatty acids is included in the diet.

*Summary.* Samples of fat from human placental, maternal, fetal, and baby tissue were examined for presence of *trans* fatty acids. While the maternal tissue contained considerable amounts of *trans* fatty acids, these lipids were not found to any measurable extent in placental, fetal, or baby fat. The results indicate that *trans* fatty acids found in human tissue must originate solely from dietary fat.

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[†] Prepuce tissue from male babies.

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# Comparative Wax Esterase Activity of Various Microorganisms.* (24484)

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Since Miyoshy's(10) demonstration that paraffin could be utilized as sole carbon source by a penicillium species, a number of reports (6,12,13) have confirmed and extended this observation. Microorganisms and higher organisms capable of breaking down beeswax (2,4,5,6,14) and tubercle wax(3,4,6,8,9,11) have been described. In view of the usefulness of wax dissolving enzymes, presumably esterases, in metabolic studies of tubercle bacilli, more exact information on occurrence and characteristics of such enzymes appeared desirable. The data here presented show that wax esterases, active against beeswax, tubercle wax and tributyrin can be demonstrated in cell free extracts from a variety of microorganisms even though esterase activity of the intact cells may be negligible.

*Materials and methods.* Organisms for study were *Micrococcus ureae* (ATCC 408), *Aspergillus flavus* (ATCC 9643), *Bacillus macerans* (ATCC 290), *Micrococcus cerolyticus* (ATCC), *Candida albicans* (isolated from clinical material from Passavant Hospital, Chicago), a penicillium species and a Gram-negative rod isolated from commercial beeswax),§ and GM1 (Gram positive rod isolated from intestinal tract of wax moth (*Galleria mellonella*).|| Stock cultures were maintained on tryticase soy agar slants. *Aspergillus flavus* and the penicillium species were grown in synthetic medium containing 1 g $NH_4NO_3$, 1 g $KH_2PO_4$, 20 mg $MgSO_4$, 0.1 g NaCl, 0.1 g $CaCl_2$, 3 mg $FeCl_3$, 1 mg biotin, 10 g dextrose, 1000 ml double distilled water. *Candida albicans*, GM1, the Gram negative rod, *Bacillus macerans* were grown in synthetic medium consisting of 6 g $(NH_4)_2HPO_4$, 0.2 g $KH_2PO_4$, 40 mg $MgCl_2$, 1 mg biotin, 10 g dextrose, 1000 ml of double distilled water. *Micrococcus ureae* and *Micrococcus cerolyticus* were grown in tryticase soy broth (BBL). To obtain sufficient growth for manometric studies a shallow layer of medium (300 ml) in a 5 liter diphtheria toxin bottle was inoculated with the growth from a slant suspended in 10 ml of culture medium. Cells in the logarithmic phase of growth were harvested by centrifugation and washed 3 times with distilled water. The cells were resuspended in distilled water and adjusted to a standard optical density which corresponded to a nitrogen content of 0.07-0.18 mg/ml depending on strain or microorganism. Nitrogen was determined by the method of Ma and Zuazaga (7). Cell extracts were prepared as follows: Cells in logarithmic growth were harvested, washed 3 times with distilled water, and resuspended in 15 ml of distilled water. Cell suspensions (containing 5-10 g wet wt) of all microorganisms except *Aspergillus flavus* and the penicillium species were subjected to sonic oscillation for 1 hour in a Raytheon 10 KC, 250 watt oscillator. To facilitate breakage of *Candida albicans* the washed cells were lyophilized and 5 times their weight of acid washed glass beads (75 $\mu$) were added. This was then resuspended in 15 ml of distilled water and subjected to sonic oscillation for 1 hour. Suspensions of *Aspergillus flavus* and the penicillium species were ground by hand for 15 minutes in a chilled mortar containing sufficient alumina 303 to produce a thick paste. Treated cell suspensions were then centrifuged at 2900 x g for 15 minutes at 5°C. The crude extracts were maintained at 5°C in an ice bath and saturated with a mixture of 95% $N_2$ and 5% $CO_2$ by bubbling in the gas mixture slowly for 5 minutes. One ml of these extracts was used per Warburg vessel and contained from 0.4 to 1.4 mg $N_2$/ml. Oxygen uptake was determined by conventional Warburg technics at 37°C. Esterase activity was deter-

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§ Submitted to American Type Culture Collection.

|| Kindly submitted by Mr. R. M. Lycette, Villa Park, Ill.

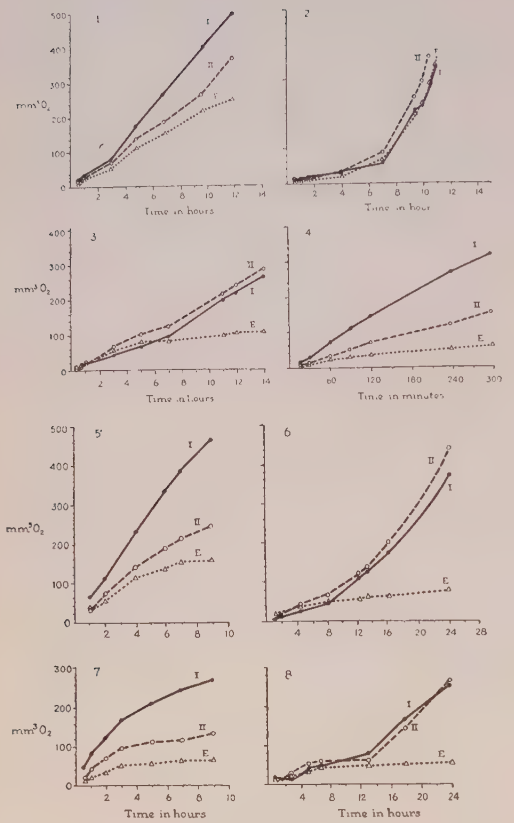

FIG. 1-8.

1, *Aspergillus flavus;* 2. Penicillium species; 3, GM1; 4, Gram negative rod; 5, *Micrococcus ureae*; 6, *Bacillus macerans;* 7, *Candida albicans*; 8, *Micrococcus cerolyticus.*

Contents of Warburg flask: 1 ml of 0.1 M potassium phosphate buffer pH 7.4, substrate, 1 ml of cell suspension containing the following amounts of nitrogen: 0.11 mg *(Aspergillus flavus)*, 0.18 mg (Penicillium species), 0.08 mg (GM1), 0.11 mg (Gram negative rod), 0.1 mg *(Micrococcus ureae)*, 0.07 mg *(Bacillus macerans)*, 0.1 mg *(Candida albicans)*, 0.08 mg *(Micrococcus cerolyticus)*. Center well contained 0.2 ml of 40% KOH. Temp. 37°C. I, Myricyl palmitate; II, TB wax; E, endogenous.

mined by measuring liberation of $CO_2$ from 0.445 M $NaHCO_3$, pH 7.9, in an atmosphere consisting of 95% $N_2$ and 5% $CO_2$, over a period of 24 hours. Myricyl palmitate (Bios), 25 mg/flask; tubercle wax (prepared by the method of Anderson, 1), 25 mg/flask; and tributyrin (Calif. Fn. for Biochem. Research), 0.2 ml/flask were used as substrates. Before use, the first 2 substrates were ground to a fine powder in a mortar immersed in dry ice. The 3 substrates appeared to contain only traces of reducing sugar (Benedict's reagent) and gave negative biuret and ninhydrin reactions.

*Results.* Oxygen consumption by washed cells in absence (endogenous) and in presence of either myricyl palmitate or tubercle wax are shown in Fig. 1-8. The microorganisms tested differed in both rate and extent to which they attacked the substrates. Higher endogenous values were observed when cells were washed with potassium phosphate buffer than with distilled water.

To determine whether initial metabolic activity of intact cells was due to release of enzyme into the environment, cell free culture broths as well as lyophilized cell free culture broths were tested. All produced negative results.

Only *Candida albicans* showed esterase activity with the substrates tested producing a $Q^N_{CO_2}$ of 92 with myricyl palmitate and 25 with tubercle wax. Negative results obtained with the other microorganisms tested were not due to lack of esterase since extracts of all microorganisms produced positive esterase activity against the wax substrates and tributyrin (Table I). No esterase activity of cell free culture broth could be demonstrated.

*Discussion.* Increase in oxygen consumption of washed suspensions of 8 cultures in presence of myricyl palmitate and tubercle wax indicated that these microorganisms possessed lipolytic enzyme systems capable of deriving energy from wax esters and it appears likely that some of these enzymes, presumably esterases, are of rather common occurrence.

TABLE I. Esterase Activity of Crude Cell Extracts of Various Microorganisms.

| | $Q^N_{CO_2}$ | | |
|---|---|---|---|
| Extract source | Tributyrin | Tubercle wax | Myricyl palmitate |
| *C. albicans* | 22 | 22 | 27 |
| *M. cerolyticus* | 71 | 10 | 27 |
| *M. ureae* | 17 | 12 | 68 |
| *B. macerans* | 1528 | 16 | 21 |
| *A. flavus* | 396 | 21 | 35 |
| Penicillium sp. | 495 | 3 | 13 |
| Gram neg. rod | 33 | 11 | 19 |
| GM1 | 58 | 20 | 36 |

Each Warburg vessel contained 1.2 ml of bicarbonate buffer pH 7.9 (1 ml in case of tributyrin), substrate and 1 ml of extract.

Pronounced lag phase in utilization of substrates by GM1, *Bacillus macerans*, and *Micrococcus cerolyticus* might suggest that adaptation occurs during this period. However, the finding of esterase activity in cell free extracts of unadapted cells speaks against this possibility. In view of the apparent absence of wax esterase in the growth medium it also seems unlikely that the lag might have been caused by the time required for autolysis to liberate esterase. The results are compatible, however, with the view that saturation with endogenous substrate of the sites of esterase or other enzymes further along the chain of reactions might have been the cause of the observed lag.

*Summary.* 1) Various microorganisms were studied for ability to metabolize myricyl palmitate (beeswax), tubercle wax, and tributyrin. All cultures except one produced positive results varying only in rate and extent to which they attacked these substrates. A pronounced lag phase in utilization of substrate by 3 microorganisms tested was attributed to saturation of enzyme sites with endogenous substrates. 2) With the exception of *Candida albicans* intact cells did not show esterase activity. However, positive results were obtained with extracts which suggested that de-esterification was an initial step in utilization of waxes as sources of energy for the various microorganisms tested.

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# Action of N,N′-diphenyl-p-phenylenediamine in Tocopherol Deficiency Diseases.* (24485)

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The ability of certain synthetic antioxidants, notably N,N′-diphenyl-p-phenylenediamine (DPPD), to delay or prevent appearance of Vit. E deficiency symptoms, including muscular dystrophy and sterility, has been demonstrated. This activity generally has been attributed to conservation of traces of Vit. E present in the lipid portion of diet and in tissues of experimental animals. In conformity with this view, Shull and associates(1) found that DPPD and other synthetic antioxidants delayed but did not prevent eventual occurrence of muscular dystrophy in guinea pigs maintained on tocopherol-deficient diet. However, experiments with rats(2) showed that fertility could be partially restored in sterile females even when Vit. E was rigorously excluded from the regenerative diet. In the present study it has been found that DPPD has no conservational effect upon Vit.

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* This investigation was supported by grant from Muscular Dystrophy Assns. of America. Vitamins were kindly supplied by Merck, Sharpe and Dohme, Rahway, N. J.

E present in liver and muscle of rabbits and rats maintained on tocopherol-deficient diet, and that the antioxidant brings about a rapid remission of symptoms of muscular dystrophy in rabbits fed a diet from which all lipoid materials have been removed.

*Procedure.* To determine the influence of DPPD on rate of depletion of tissue tocopherols, twenty-two 6-weeks-old rabbits were placed on tocopherol-low basal diet of the following composition: Labco casein 20, Cerelose 20.4, cornstarch 40, molecular-distilled lard 10, salts 446(3) 4, cellulose 5, Vit. premix(4) 0.5, choline chloride 0.1. Vit. A and D were provided at concentrations of 10,000 I.U. and 1000 I.U./kg of diet, respectively. During 2-week preliminary period the diet was supplemented with $\alpha$-tocopheryl acetate at 1 g/kg. At this time 4 animals were sacrificed and the remainder were divided into 2 groups of 9 each. One group was placed on basal tocopherol-low diet; the other received the same diet to which DPPD was added at 0.005%. Additional animals were sacrificed from each group at intervals of approximately 3 weeks. Livers and segments of thigh skeletal muscle were removed and frozen for Vit. E analysis. A similar experiment was carried out using 30 weanling rats. These animals were maintained for 2 weeks on tocopherol-low basal diet described elsewhere(4) plus a supplement of 30 mg $\alpha$-tocopheryl acetate/week. Then 6 rats were sacrificed and the remainder divided into 2 groups which were placed on basal diet with or without 0.001% DPPD. Additional animals were killed at intervals for liver and muscle analyses. Livers were ground with anhydrous sodium sulfate until dry. 10 g muscle samples were freed of extraneous connective tissue, ground with acid-washed sand and dried with sodium sulfate. The dried samples were placed in Erlenmeyer flasks and extracted with peroxide-free ether overnight in mechanical shaker. After filtration the ether was removed by warming in water bath under jet of nitrogen and saponification was carried out in the dark in the presence of pyrogallol according to slight modification of procedure of Eggitt and Ward(5). Sterols were removed from the non-saponifiable fraction of liver samples by overnight crystallization from ethanol in the cold. After evaporating the solvent under nitrogen, carotenoids and Vit. A were removed from liver extracts by dissolving the residue in benzene and passing through a column of Floridin XXS previously treated with stannous chloride in HCl as described by Beckmann(6). Finally, the benzene was evaporated in nitrogen atmosphere and the residue dissolved in ethanol for determination of Vit. E by the Emmerie-Engel reaction. 5 $\mu$g per sample could be determined readily by this procedure. In accord with the fact that $\alpha$-tocopheryl acetate was the only dietary form used, chromatography on paper, using the systems of Green *et al.*(7), failed to detect any of the other natural isomers. The curative activity of DPPD was tested on dystrophic rabbits maintained on a lipid-free, tocopherol-free diet. Dystrophy was induced by feeding the diet of Young and Dinning(8) except that salts 446(3) and a different vitamin mixture were used(4). When distinct symptoms appeared, the dystrophic animals were placed on basal diet of the following composition: Labco casein 15, sucrose 43.9, cornstarch 36, salts 446(3) 4, choline chloride 0.1, vitamin premix in Cerelose(4) 1. The short recovery period required for regeneration of dystrophic rabbits with Vit. E permits removal of all lipids from the diet without risking a deficiency of essential fatty acids prior to recovery. The presence of 3% cod liver oil in the depletion diet also assured an adequate storage of Vit. A and D. The following supplements were administered daily to different groups of dystrophic animals: 10 mg $\alpha$-tocopheryl acetate; 20 mg DPPD; none. The supplements were suspended in 1 ml glycerol containing 0.01 ml Tween 80. Negative controls received the carrier alone. Twenty-four hour urine collections were made at intervals for determination of total and preformed creatinine by modification of the method of Hare(9).

*Results.* The effect of feeding a Vit. E-deficient diet on tocopherol levels in tissues of rabbits and rats, and the influence of DPPD, are illustrated in Fig. 1. DPPD effected no conservation of Vit. E in tissues of either species, and such an effect does not,

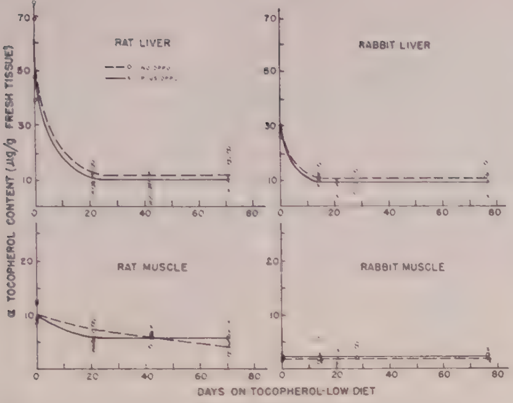

FIG. 1. Influence of DPPD on α-tocopherol concentration in tissues of rabbits and rats fed a Vit. E-deficient diet.

therefore, account for its ability to replace the vitamin in the diet. It is also noteworthy that the tocopherol level of skeletal muscle of rabbit is apparently unaffected by dietary depletion. This observation is in accord with that of Blaxter(10) who found that Vit. E content of muscle of dystrophic calves was at least as great as that of normal animals. The small decline noted in rat muscle tocopherol is of doubtful significance. It appears that the turnover of tocopherol in skeletal muscle of both species is exceedingly slow, and that after a period of relatively rapid depletion of "labile" tocopherol in the liver, the concentration in this organ also reaches a plateau which is maintained for extended periods. The rats made good growth during experiment while rabbits exhibited the customary slow gains observed with purified diets. Histological examination of skeletal muscle of rabbits maintained on basal diet for 10 weeks revealed evidence of mild dystrophy. Muscular degeneration on this diet requires a much longer period of depletion than is required when high levels of unsaturated fatty acids are ingested.

Five dystrophic rabbits maintained on lipid-free diet and treated with 20 mg DPPD daily showed symptomatic evidence of improvement in 2 to 3 days and a disappearance of symptoms in 5 to 6 days. These animals also underwent a remission of creatinuria, as indicated by creatine:creatinine ratios which declined from pretreatment range of 2.21-5.01 to 0.09-0.42 after treatment for 5 days. This group was continued on DPPD supplementation for 15 days, during which time growth was resumed and condition of the animals markedly improved. Terminal urine collections yielded creatine:creatinine ratios of 0.12-0.33. The condition of 3 control animals given Tween-glycerol carrier, deteriorated rapidly and they succumbed in 4 to 8 days. Three dystrophic animals treated with Vit. E. recovered in a manner similar to that described for the DPPD treatment.

These results demonstrate that DPPD possesses curative activity with respect to muscular dystrophy that is independent of the presence of Vit. E in the diet. Earlier observations(2) indicating that DPPD is also active in rehabilitation of sterile female rats have been confirmed in more recent experiments. Partial regeneration was obtained following administration of 0.005% DPPD in Vit. E-free diet in which a high-purity methyl linoleate-urea complex(11) served as the source of essential fatty acids and fat-soluble vitamins were provided in the crystalline state. Reproductive performance of rats fed DPPD is always inferior to that observed using Vit. E, however, owing to the high order of toxicity of the synthetic compound for pregnant females.

These findings support the view that biological activity of the tocopherols with respect to muscular dystrophy is associated with their antioxygenic potency, and are in accord with known activity of α-tocopherylhydroquinone and α-tocopherylquinone. The efficacy of DPPD with respect to rat sterility is, however, in contrast to the reported inactivity of these oxidation products of α-tocopherol, and implies that this syndrome is also a consequence of an inadequacy of suitable antioxygenic compounds. Moreover, the hydroquinone has been reported to have activity comparable to α-tocopheryl acetate in reversing testicular damage in Vit. E deficient hamsters (12). It is of interest that antioxygenic potencies of α- β- and γ-tocopherols, as determined in storage tests conducted at temperatures near the physiological range using carotene and ethyl oleate, are in the same order as their biological activities(13).

Tests of ability of DPPD to reactivate isooctane-extracted DPNH-cytochrome c reductase in the Keilin-Hartree heart muscle preparation have confirmed the finding of Nason and Lehman(14) that this antioxidant is ineffective in replacing α-tocopherol. However, serum albumin was found to be an unsatisfactory agent for suspending DPPD in this system and other materials are being investigated.

Curative activity of DPPD with respect to muscular dystrophy in the rabbit is in contrast to its reported inability to prevent hypercholesterolemia and muscular degeneration in guinea pigs fed a tocopherol-low diet (15). An explanation of this apparent discrepancy must await further investigation.

*Summary.* Administration of DPPD to growing rabbits and rats fed a Vit. E-low diet does not modify rate of depletion of vitamin from the liver. Tocopherol content of skeletal muscle was essentially unaffected by dietary restriction. DPPD was as effective as α-tocopherol, at level administered, in bringing about a remission of muscular dystrophy and creatinuria in rabbits maintained on diet devoid of tocopherols. Ability of DPPD to partially regenerate sterile female rats maintained on Vit. E-free diet was confirmed. These findings indicate that the effectiveness of DPPD as a substitute for Vit. E is not due to conservation of the vitamin in tissues or to the presence of residual tocopherols in the diet.

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# Conversion of Serotonin (5-Hydroxytryptamine) to 5-Hydroxyindoleacetic Acid by Rabbit Blood. (24486)

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Serotonin (5-hydroxytryptamine) is oxidized to 5-hydroxyindoleacetic acid (5-HIAA) in animals and man(1). The latter compound is excreted in the urine. The catalyst for oxidation of serotonin is thought to be monamine oxidase. Although many tissues contain this enzyme, liver is usually the most active source. Werle and Roewer(2) have reported an amine oxidase present in dog blood. In other species, however, blood has been considered to be lacking in this enzyme activity(3). Tabor *et al.* (4) found an amine oxidase in beef plasma, but substrate and inhibition specificities differed markedly from those described for the monamine oxidase in liver. Reserpine causes a release of serotonin and histamine from platelets *in vivo*(5). *In vitro* studies have shown that incubation of reserpine with rabbit platelets suspended in plasma also liberates serotonin and histamine. However, after in-

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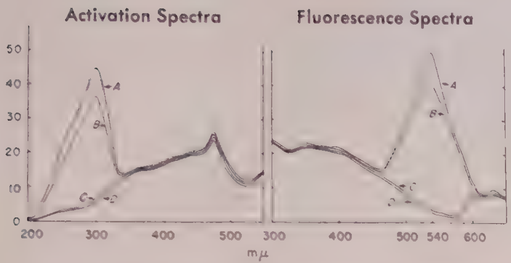

FIG. 1. Activation and fluorescence spectra in strong acid for 5-HIAA from (A) 3 ml of plasma after incubation of whole blood with reserpine, (B) 5 γ of authentic 5-HIAA, (C) 3 ml of control plasma, and (D) blank.

cubation of reserpine with rabbit whole blood, histamine was increased in the plasma, but serotonin was not.† Subsequent investigation has shown that serotonin is oxidized to 5-HIAA in presence of rabbit blood.

*Material and methods.* Blood was obtained from rabbits by cardiac puncture. All glassware used in handling blood or platelets was siliconized. The anticoagulant was disodium ethylenediaminetetraacetate (EDTA), 10% by volume of 1% solution in n-saline. Platelet rich plasma was obtained by the method of Dillard *et al.*(6). Red blood cells, suspended at bottom of centrifuge tube after complete removal of platelet layer by suction, were used for red blood cell studies. Although reduced in number, white blood cells, as shown by smear, were still present. Serotonin and histamine were determined following procedure of Weissbach *et al.*(7). By this method, both total 5-hydroxyindole compounds and also serotonin can be measured in the same blood or tissue aliquot. The 5-HIAA in plasma was assayed by modification of colorimetric method of Udenfriend *et al.*(1) for urine. The 5-HIAA was determined by measuring its fluorescence in 1 ml of phosphate buffer (pH 7) or after addition of strong acid to the buffer. Activation wavelength for 5-HIAA in both solutions is 300 mμ but the fluorescence wavelength in neutral or weakly acid solution is 350 mμ and in strongly acid solution is 540 mμ. In neutral or weakly acid solutions fluorescence is greater but less specific, with biologic materials, than when strong acid is used. In plasma, other material was found to fluoresce near 350 mμ at pH 7; at 540 mμ no interfering substance was apparent (Fig. 1). For all determinations of 5-HIAA in this study fluorescence was measured in strong acid at 540 mμ. Serotonin, as much as 100γ, did not produce a significant reading when assayed by the procedure for 5-HIAA.

*Results.* 1. Incubation of rabbit whole blood with reserpine. For experimental samples, 6 ml of blood were mixed with reserpine solution (20γ/ml in 5% dextrose in water) to make final dilution 0.3γ/ml; control samples were diluted with the same volume of 5% dextrose in water. After incubation at 37°C, whole blood determinations were done for total 5-hydroxyindoles, serotonin and histamine.

TABLE I. Total 5-Hydroxyindoles, Serotonin, and Histamine after Incubation of Rabbit Blood with Reserpine.

| | Whole blood (γ/ml) | | | | | |
|---|---|---|---|---|---|---|
| | 5-Hydroxyindole compounds | | | | Histamine | |
| Incubation time (hr) | Control | | Exp. | | Control | Exp. |
| | Total | Serotonin | Total | Serotonin | | |
| 3.0 | 3.1 | 3.2 | 3.0 | 2.8 | 2.0 | 2.0 |
| | 4.2 | 4.3 | 4.1 | 3.8 | 2.2 | 2.1 |
| 3.5 | 4.1 | 4.0 | 4.0 | 3.5 | 1.2 | 1.2 |
| | 5.2 | 5.3 | 4.6 | 4.4 | 1.5 | 1.7 |
| 4.0 | 3.5 | 3.6 | 3.1 | 2.8 | 2.0 | 2.0 |
| 4.5 | 4.0 | 4.3 | 3.5 | 3.1 | 1.2 | 1.2 |
| | 4.1 | 4.1 | 3.5 | 2.4 | 1.6 | 1.5 |
| 5.0 | 4.6 | 4.8 | 3.7 | 2.9 | 3.1 | 3.0 |
| | 4.5 | 4.5 | 3.5 | 2.3 | 3.0 | 2.7 |
| 16.0 | 5.0 | 5.0 | 3.8 | .2 | 3.7 | 3.4 |
| | 4.5 | 4.2 | 3.6 | .3 | 2.1 | 1.8 |
| | 4.5 | 4.2 | 3.3 | .9 | 3.0 | 2.7 |
| | 4.2 | 4.0 | 3.4 | .8 | 2.1 | 2.4 |

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† Unpublished observations.

TABLE II. Plasma 5-HIAA and Serotonin after Incubation of Rabbit Whole Blood with Reserpine.

| | 5-HIAA (γ/ml) | | Serotonin (γ/ml) | | | |
|---|---|---|---|---|---|---|
| | Plasma | | Plasma | | Whole blood | |
| Incubation time (hr) | Control | Exp. | Control | Exp. | Control | Exp. |
| 5.0 | .2 | 1.4 | .2 | .2 | 6.0 | 3.5 |
| | .2 | 1.9 | .1 | .1 | 5.8 | 3.3 |
| | .1 | .7 | .1 | .1 | 3.5 | 1.8 |
| | .1 | 1.2 | .2 | .2 | 5.0 | 2.7 |
| 16.0 | .2 | 1.8 | .2 | .1 | 4.7 | .4 |
| | .2 | 2.5 | .1 | .1 | 3.3 | .4 |
| | .3 | 4.2 | .1 | .1 | 4.8 | .9 |
| | .2 | 2.5 | .1 | .1 | 2.4 | .4 |

The results (Table I) show that amount of histamine/ml of blood was the same in control and experimental blood after incubation. However, the amount of serotonin in experimental blood was less than that in the control samples. The greatest difference was noted after 16 hours. Total 5-hydroxyindoles decreased slightly. Plasma content of serotonin was essentially the same in control and experimental samples (Table II). The evidence suggested that serotonin was liberated during incubation with reserpine and was converted to some other 5-hydroxyindole compound. When air over the blood was displaced by nitrogen, no change in final result was noted.

2. Assay for serotonin and 5-HIAA. Whole blood and plasma serotonin were determined, and in addition, plasma 5-HIAA. No 5-HIAA or other 5-hydroxyindole compounds except serotonin have been found in normal rabbit blood(8). The results given in Table II show that serotonin must be released and converted to 5-HIAA or a 5-HIAA-like compound. When whole blood was also incubated with serotonin (5γ/ml), with and without reserpine, for 5 hours, 5-HIAA was found in the plasma.

3. Identification of 5-HIAA. Fig. 1 gives fluorescence characteristics of authentic 5-HIAA, run through assay procedure, and of that isolated from plasma. The plasma from 10 ml of whole blood, incubated 5 hours with 1 mg of serotonin, was treated as for the 5-HIAA assay. However, the ether used for extraction was evaporated to a small volume and transferred to Whatman #1 paper for chromatography. The developing solvent was n-propanol 1 N amonia (5:1). After drying and spraying with 0.1 N HCl, pink fluorescent spots under ultraviolet light were seen for authentic 5-HIAA at $R_f$ 0.21, for the experimental sample at $R_f$ 0.22, and for standard serotonin at $R_f$ 0.57. Ehrlich's reagent produced a blue green spot at $R_f$ 0.21 for authentic 5-HIAA and at $R_f$ 0.22 for the compound isolated from blood.

4. Part of blood responsible for oxidation. Platelets broken by shaking with water, normal rabbit plasma, and plasma from blood warmed with reserpine at 37°C for 12 hours were incubated as previously described(9) to determine presence of monamine oxidase activity. After 2 hours, the mixtures were assayed for serotonin and 5-HIAA. No conversion to 5-HIAA was found. Serotonin (2γ/ml) in n-saline adjusted to pH 7 with phosphate buffer and incubated, with or without reserpine, for as long as 12 hours, showed essentially no change in serotonin content. When serotonin, and histamine, were added to normal rabbit plasma, and incubated at 37°C 12 hours, destruction of both serotonin and histamine occurred (Table III). However, no conversion of serotonin to 5-HIAA was found by comparing amount of total 5-hydroxyindoles with the amount of serotonin present. Reserpine had no apparent effect on this reaction. When 5-HIAA was incubated with plasma for periods to 8 hours, no destruction of the acid was noted. Platelet rich plasma, incubated with serotonin 3 to 5 hours, with or without reserpine, did not yield 5-HIAA. Upon incubation of the red blood cell layer, obtained by differential centrifugation of blood, with serotonin (5γ/ml in n-saline) for 3 hours at 37°C in Dubnoff shaker, 5-HIAA

TABLE III. Incubation of Serotonin and Histamine with Normal Rabbit Plasma; Effect of Reserpine.*

| Incubation time (hr) | Plasma (γ/ml) Total 5-hydroxyindoles | | Serotonin | | Histamine | |
|---|---|---|---|---|---|---|
| | Reserpine | Control | Reserpine | Control | Reserpine | Control |
| 0 | 3.6 | 3.6 | 3.7 | 3.6 | 3.1 | 3.0 |
| 1 | 3.3 | 3.4 | 3.6 | 3.4 | 2.5 | 2.6 |
| 3 | 2.7 | 2.7 | 2.6 | 2.5 | 1.9 | 1.7 |
| 6 | 2.0 | 1.9 | 2.1 | 2.2 | 1.6 | 1.5 |
| 12 | .6 | .7 | .7 | .6 | .8 | .7 |

* .3 γ/ml of plasma.

was found in the plasma. Addition of reserpine did not alter amount of final acid to any extent.

5. Marsilid (1-isonicotinyl 2-isopropyl hydrazide) *in vivo*. Marsilid is a potent inhibitor of monamine oxidase activity *in vitro* and *in vivo*(10). Blood was obtained from 3 rabbits before and 2 hours after being given 200 mg/kg of marsilid intraperitoneally. Blood was incubated with reserpine 5 hours, as before, and plasma analyzed for 5-HIAA and serotonin. The results for those samples drawn before marsilid injection were similar to results given in Table II. However, for those samples drawn after marsilid injection, control and experimental plasmas had the same average amount, 0.2γ/ml, of 5-HIAA, while average amount of serotonin in experimental plasmas was 1.0γ/ml and in control plasmas was 0.1γ/ml. These results indicate that serotonin was released by reserpine but not converted to 5-HIAA in the blood after marsilid treatment.

*Discussion.* In the present study, free serotonin was metabolized by rabbit whole blood. The product was thought to be 5-HIAA as shown by paper chromatography and by its fluorescence characteristics. Platelets did not oxidize serotonin to 5-HIAA. Both serotonin and histamine were destroyed slowly by normal rabbit plasma. However, serotonin was not converted to 5-HIAA by the plasma. Normal blood plasma has been stated to have an extremely low diamine oxidase activity(11), and diamine oxidase has been reported to attack monamines(12).

Serotonin, added to red blood cells separated by differential centrifugation from platelets, was converted, in part, to 5-HIAA. The red cell suspension was not entirely free of white blood cells. Consequently, red, or possibly white, blood cells must be involved in conversion of serotonin to 5-HIAA. Blood from rabbits pretreated with marsilid did not oxidize serotonin to 5-HIAA. The evidence suggested that the red blood cell, or possibly the white cell, contains an amine oxidase. Further evidence that the enzyme was probably a monamine oxidase was given by the fact that histamine was not destroyed in rabbit whole blood. Reserpine, except for its ability to release serotonin from platelets to be acted upon by the oxidative system did not appear to effect the conversion. The significance of a monamine oxidase in the cellular elements of blood is not known.

*Summary.* Free serotonin, in the presence of rabbit whole blood, is oxidized to 5-HIAA. The latter was identified by paper chromatography and by its fluorescence characteristics. Platelets or plasma do not produce the reaction. Evidence is presented that red blood cells, or possibly the white cells, are factors in the conversion of serotonin to 5-HIAA.

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## Fluid and Electrolyte Movement Across Intestinal Wall of Bullfrog. (24487)

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While normal movement of fluid and electrolyte across intestinal wall is one of net absorption, experimental circumstances, both *in vitro* as well as *in vivo,* frequently do not result in absorption. The following results indicate that sacs of bullfrog intestine do absorb saline and establish concomitant gradients of chloride and bicarbonate ion.

*Methods.* Large bullfrogs (*Rana catesbiana*) were maintained in a state of pseudahibernation at 12°C. Sacs of mid-small intestine (jejunum) were formed by taking segments 2-3 cm in length from the pithed frog and tying each end. The large intestine was tied at the ends to form a sac. In half of the experiments, normal orientation of the sac wall was retained with mucosal surface bounding the lumen. The remainder of the sacs were formed after first everting the intestinal tube. The lumen of the everted sacs was thus bounded by the serosal surface. The sacs were incubated at room temperature (24°C) in 5 ml of a salt solution, pH 7.4, containing 87 mM NaCl, 20 mM $NaHCO_3$, 2.5 mM KCl, 1.25 mM $CaCl_2$, 0.5 mM $MgSO_4$, 1.33 mM $Na_2HPO_4$, 0.33 mM $NaH_2PO_4$ and 5 mM glucose/liter. About 0.3-1.3 ml of the same solution was placed inside each sac. 95% $O_2$ and 5% $CO_2$ was bubbled through Dubnoff shaker during experimental period of 4-6 hours. Water movement was measured by weighing the sacs at time "zero" (after 10-15 minute equilibration period) and at end of experiment, 4-6 hours later. Adherent water was removed from the sacs prior to weighing by rolling them on a dry glass surface. This resulted in a uniform stable weight. "Drying" the intestinal sacs by blotting them on a variety of papers did not result in a reproducible weight. Successive drying on filter paper was accompanied by small progressive decrease in weight. In early experiments, radio-inulin ($C^{14}$) was incorporated in the solution placed inside the sacs. Radio-inulin concentration within the sacs as well as in the bathing solution was determined(1) at end of experiment. The change of concentration of radio-inulin in the solution within the lumen of the normally oriented sacs indicated that weight change did measure net movement of water across intestinal wall. The bicarbonate concentration of inner contents of sacs was measured by back-titration, with NaOH, of an added volume of a standard HCl solution. Chloride concentrations of inner contents were determined by coulometric - amperometric method of Cotlove, *et al.*(2).

*Results.* The tabulated results indicate that there is a net movement, corresponding to absorption, of both water and electrolyte across wall of the normally oriented and everted sacs of small and large intestine.

Use of the everted sac has been favored in the study of isolated mammalian intestine because of the presumed better oxygenation of the epithelial surface(3). In our experiments, there was a significantly greater movement of water across the wall of the everted sac than across that of normally oriented sacs. However, while there was no movement of radio-

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TABLE I.

| | Net movement* | Conc. change, meq/l | Net movement* | Conc. change, meq/l |
|---|---|---|---|---|
| | Small intestine | | | |
| | Normal sacs (12) | | Everted sacs (9) | |
| $H_2O$ | $-8.0 \pm 2.0$ | | $+32.0 \pm 5.0$ | |
| $Cl^-$ | $-1.9 \pm .4$ | $-20.1$ | $+1.4 \pm .5$ | $+6.5$ |
| $HCO_3^-$ | $+1.3 \pm 1.8$ | $+4.8$ | | |
| | Large intestine | | | |
| | Normal sacs (10) | | Everted sacs (10) | |
| $H_2O$ | $-.4 \pm .1$ | | $+5.0 \pm 1.0$ | |
| $Cl^-$ | $-1.1 \pm .2$ | $-22.0$ | $+.5 \pm .06$ | $+6.6$ |
| $HCO_3^-$ | $-.5 \pm .04$ | $-5.0$ | $+.9 \pm .2$ | $+4.2$ |

* Net movement into or out of the sac for $H_2O$ in μl and for ions in μeq/hr/100 mg wet wt of tissue.

± signifies stand. error, and figure in parenthesis No. of experiments.

inulin out of the normal sacs, there was a decided loss of radio-inulin from the everted sacs of small intestine. In 8 experiments, mean internal radio-inulin content decreased due to diffusion by 15.6% and 1.1% in the everted sacs of small and large intestine, respectively. Thus, a rapid transfer of water occurred in spite of an unusual permeability to a relatively large molecule, radio-inulin. It is not known whether the greater transfer of water resulted from the more efficient oxygenation of epithelial cells or from a more rapid hydraulic flow through larger pores.

Presumably the escape of inulin out of the everted sacs is an experimental artifact which could have resulted from either excessive stretching during eversion or from the physical trauma associated with rocking the small sacs within 10 ml breakers. In the mammalian small intestine impermeability to radio-inulin has been clearly demonstrated.

The difference between everted and normal sacs is also evident in concentration changes developed. These were less in the everted sacs.

It is noteworthy that in 6 experiments the transfer of water was not affected by absence of glucose from the solution within and without the sacs. This is in agreement with the finding of Lifson and Parsons(5) that glucose transport was not a necessary prerequisite for water transport by the isolated rat intestine, even though exogenous glucose utilization was necessary.

While ionic absorption by the canine jejunum has been considered to be a relatively inefficient process(6), absorption from the normally oriented bullfrog jejunal sacs resulted in an appreciable concentration gradient of chloride. Under comparable conditions the electrical potential across the small intestine is close to zero(unpublished). The accumulation of bicarbonate within the lumen of normally oriented small intestine sacs of the frog is similar to the transfer occurring in the hamster(7). In contrast, bicarbonate is depleted at the mucosal surface in normal man, intact dog(8) and the isolated rat small intestine(7).

The transfer of chloride by the normally oriented large intestine sacs of the frog created a striking concentration gradient for chloride. In the case of bullfrog large intestine there was also a depletion of bicarbonate at the mucosal surface. While this would be expected in view of the electrical potential difference developed by active sodium transport across the isolated bullfrog large intestine mucosa(9), it contrasts sharply with bicarbonate accumulation that occurs in large volumes of saline placed in the colon of man and dog(10). In the one instance where sufficiently fluid contents could be aspirated from the colon of a freshly killed bullfrog, the pH was 7.41.

*Summary.* 1) Isolated sacs of small and large intestine of the bullfrog incubated at room temperature, transfer water and electrolyte from mucosa to serosa. The transfer of chloride results not only in a net movement but also in creation of concentration gradients which are pronounced in the case of both small and large intestine. There is an increase in bicarbonate concentratiion at the mucosal surface of the small intestine and a decrease in bicarbonate concentration at the mucosal surface of the large intestine. 2) Eversion of the intestines resulted in sacs that were significantly permeable to radio-inulin. Associated with this abnormal permeability, there is a greater transfer of water and smaller ionic concentration gradients develop.

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## "Dopa Oxidase" in Melanocytes of X-Irradiated Quiescent (*Telogen*) Hair Follicles. (24488)

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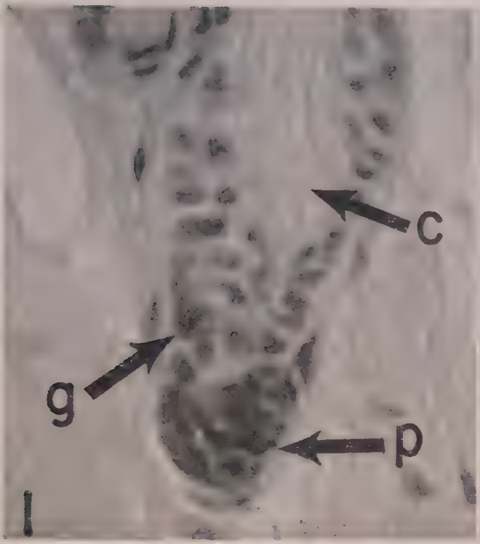
Melanocytes within growing hair follicles of pigmented mice contain melanin granules and active "dopa oxidase." In the presence of dihydroxyphenylalanine (dopa) *in vitro*, they form abundant dopa-melanin(1). On the other hand, melanocytes of quiescent (telogen) hair follicles in pigmented mice generally are unpigmented and are dopa negative (1). Thus, it has not been possible to identify melanocytes with certainty at resting stage of hair growth cycle. Although X-radiation has been shown, under certain conditions, to stimulate melanogenic activity within latent melanocytes of the epidermis, there is no report of a similar effect on latent melanocytes within quiescent hair follicles. Previous studies have shown that repeated partial-body exposure of mice to 200 r/week of X-radiation is particularly effective in eliciting epidermal hyperpigmentation(2). Utilizing similar technics in the present study, mice with quiescent hair follicles were exposed weekly to X-radiation. The purpose was 2-fold; first, to determine whether "dopa oxidase" activity could be demonstrated in latent follicular melanocytes following X-irradiation; second, to characterize more fully the distribution of melanocytes within telogen hair follicles.

*Materials and methods.* Eight C3H mice (4 males, 4 females), 4 to 8 months of age, were shielded with lead, except for small rectangular area (1.5 x 2.0 cm) on the dorsum, and exposed to 200 r of X-rays once weekly for 3 or 4 weeks. The radiation factors were: 100 kv, 5 ma, 0.25-mm Al, HVL-0.55 mm Al, target distance, 15 cm, and dose rate, 580 r/minute. The design of the lead shield ensured irradiation of essentially the same area of skin each week. At start of irradiation, hair follicles of all mice were quiescent (telogen stage) as indicated by light pink color and thinness of skin(3,4). Subsequently, gross examinations of skin of each mouse were made at frequent intervals to detect alterations in hair growth status. The mice were killed by cervical dislocation 24 hours after last exposure to radiation. Specimens of skin were removed from both irradiated and non-irradiated regions of the dorsum and tested for "dopa oxidase" activity according to the method of Becker(5). The specimens were then dehydrated in dioxane and imbedded in paraffin; sections were cut at 8 $\mu$ and stained with Mayer's alcoholic carmine. Skin samples from 4 non-irradiated C3H mice with quiescent hair were tested for "dopa oxidase" activity in a similar manner.

*Results.* Due to lack of pigmentation, melanocytes could not be identified in quiescent hair follicles of non-irradiated mice, although pigmentary debris was frequently observed. "Dopa oxidase" activity was also absent in melanocytes of non-irradiated telogen



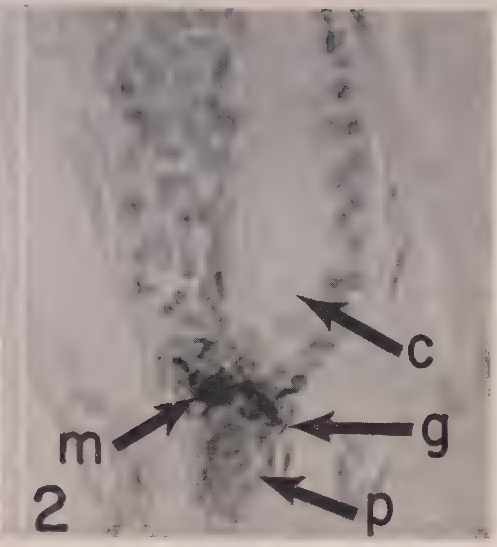


FIG. 1. Non-irradiated telogen hair follicle following treatment with dopa reagent. Note absence of dopa reactive cells. ×450.

FIG. 2. X-irradiated telogen hair follicle following incubation in dopa reagent. Two dopa reactive dendritic melanocytes are evident in the epithelial germ region. ×450.

Key: c = hair club; g = epithelial germ; p = dermal papilla; m = melanocyte.

hair follicles; no specific cellular darkening was observed following treatment with dopa reagent (Fig. 1). In 5 of 8 irradiated mice the hair follicles in exposed areas of skin remained quiescent throughout 3 or 4 week period of irradiation. There was no indication of epilation to gross inspection. In cases where growing hair was observed in the irradiated regions it was apparently associated with normal waves of hair growth rather than elicited by the radiation process. In contrast to non-irradiated quiescent hair follicles, telogen hair follicles in exposed areas of all 5 irradiated mice contained dopa reactive dendritic cells in region of hair germ, indicative of the presence of "dopa oxidase" (Fig. 2). The dendritic processes of intensely pigmented melanocytes were insinuated between non-reactive epithelial cells of the germ region. No dopa reactive cells were observed in dermal papilla (Fig. 2). Hair follicles from shielded regions of irradiated mice contained no dopa reactive cells, with the exception of a few hair follicles close to the border of the shielded areas where slight overlapping may have occurred during the repeated exposure to radiation.

*Discussion.* It has been reported previously that "dopa oxidase" activity is absent in melanocytes of telogen hair follicles(1). The results of our study confirm this observation and further demonstrate that "dopa oxidase" activity may be released by X-irradiation of quiescent hair follicles. The chemical nature of radiation-sensitive anti-melanogenic factor in telogen hair follicles of mice is as yet uncharacterized. It has been shown, in other species, that sulfhydryl compounds inhibit melanogenesis in skin and that the inhibitory effect is lost following treatment with ultraviolet rays(6). Whatever its nature, during normal hair growth cycles, the inhibitory factor must be lost shortly after new hair growth starts since follicular melanocytes then synthesize pigment granules and react positively toward dopa reagent(1).

The fate of melanocytes in telogen hair follicles of mammals is controversial, in large part due to difficulty of identifying melanocytes during this stage of hair growth activity. Thus, it has been suggested that at telogen stage melanocytes are localized in the epithelial germ regions of hair follicles in mice and rats(7,8) and in dermal papillae of humans and dogs(9,10). In our study the observation that dopa reactive dendritic cells were restricted to the epithelial germ regions of X-irradiated telogen hair follicles in mice supports the former view. Although it is quite possible that species differences exist in this regard, radiation technics utilized in our work afford a means for critical analysis of these apparently discordant viewpoints.

*Summary.* "Dopa oxidase" activity was absent in melanocytes of telogen hair follicles of non-irradiated mice. Following partial-body exposure to 200 r/week of X-radiation for 3 or 4 weeks, numerous dopa reactive dendritic cells were observed in the epithelial germ regions of irradiated quiescent hair follicles. Cells of the dermal papillae in irradiated and non-irradiated telogen hair follicles failed to exhibit "dopa oxidase" activity. The results thus support the view that melanocytes normally are restricted to the epithelial germ regions of hair follicles during telogen stage of hair growth cycle.

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## Rabbit Endometrial Response to Intravenously Administered Progesterone.

(24489)

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Spectrophotometric observations concerned with intravenously administered progesterone have revealed rapid clearance of the hormone from the ether soluble fraction of rabbit and human blood serum(1,2). The results were constant whether the vehicle in which progesterone was administered was saline, ethanol, propylene glycol or a mixture of ethyl carbamide, ethyl urethane and propylene glycol.* It has been suggested that disappearance of progesterone from the ether soluble fraction of blood serum resulted from tissue storage of this substance and continuing biological activity potential. It is, however, equally possible that rapid clearance of progesterone from blood serum resulted from rapid metabolism of the hormone. These experiments were planned to establish the biologic effectiveness of intravenous progesterone. Duration and intensity of tissue response to intravenous progesterone would indicate degree of storage and rate of destruction of the hormone.

*Methods.* Immature female rabbits, average weight 1200 g, were injected with 100 I.U. of estrogenic substance a day for 6 days.† Twenty-four hours after last injection of estrogen, intravenous progesterone as Proluton I. V., was administered into marginal ear vein of the rabbit. Twenty-four to 168 hours following intravenous injection, the rabbits were sacrificed. Ovaries were inspected and biopsied for evidence of activity. Uteri were removed and portions prepared for microscopic study. The Corner-Allen(3) progestational proliferation response and endometrial epithelial mitotic counts were utilized as evidence of luteoid activity within the endo-

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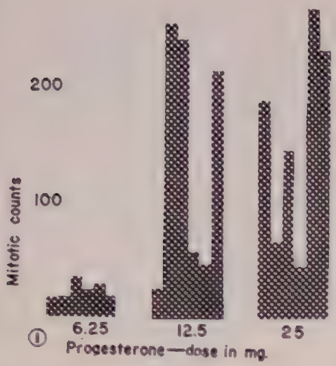
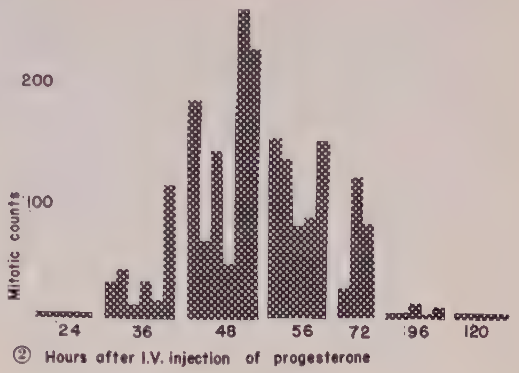
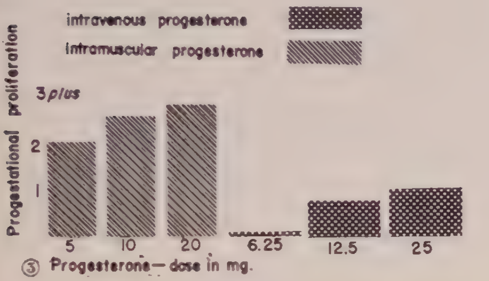
[*] Progesterone dissolved in ethyl carbamide, ethyl urethane and propylene glycol was generously supplied by Schering, A. G. of Berlin, Germany as PROLUTON I.V.

[†] The estrogenic substance was generously supplied by Parke-Davis as THEELIN.







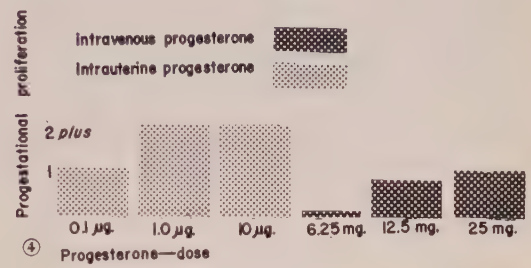


FIG. 1. Epithelial mitotic figure count per total cross section of the rabbit endometrium at 48 hr after a single intrav. inj. of progesterone.

FIG. 2. Epithelial mitotic figure count per total cross section of rabbit uterus induced by single intrav. inj. of progesterone.

FIG. 3. Comparison of Corner-Allen progestational proliferation induced in rabbit uterus by single intramusc. and intrav. inj. of progesterone; 48 hr after administration.

FIG. 4. Comparison of Corner-Allen progestational proliferation induced in rabbit uterus by single intrauterine and intrav. inj. of progesterone; 48 hr after administration.

metrium. Proliferation responses were recorded from 1+ to 4+. Mitotic counts are listed as total counts/cross section.

*Results.* Previous studies have shown that intravenous dose of 25 mg of progesterone, in rabbits of the size used, will produce saturation of rabbit blood serum with the hormone (2). The saturation concentration of 30 µg of progesterone/ml of blood serum is greater than the physiologic level of the hormone in the rabbit. Consequently, smaller than saturation doses of progesterone were administered in early phases of the study. Doses of 1 mg, 2 mg, and 5 mg produced no evidence of progestational activity either when judged by the Corner-Allen progestational proliferation index or the more sensitive epithelial mitotic count.

Total dosage levels of 6.25 mg, 12.5 mg and 25 mg of progesterone were used. Mitotic counts observed in animals receiving these dosage levels are shown in Fig. 1. The maximum mitotic figure count is obtained with 12.5 mg of progesterone. Doubling of this to the saturation dose makes no apparent difference in total mitotic figure counts. Injection of 6.25 mg of progesterone yields a minimum epithelial mitotic count.

The Corner-Allen progestational proliferation response of 32 test animals with saturation dose of 25 mg of intravenous progesterone was noted. The maximum observed response of 1+ progestational proliferation became apparent 48 hours after injection and remained so through 144 hours after injection. At 168 hours following intravenous administration of progesterone, the endometrium had returned to a resting state.

Mitotic figure counts in the same group of animals are illustrated in Fig. 2. The first evidence of increased endometrial mitotic activity was found at 36 hours after injection.

Maximum activity was found at 48 hours after intravenous injection with a gradual return to control levels at 96 hours after intravenous injection of progesterone.

In Fig. 3 progestational proliferation induced by single intramuscular injection of progesterone(4) is compared to proliferation following intravenous progesterone. These comparisons are at 48 hours after administration of the hormone. Saturation doses of intravenous progesterone are incapable of producing progestational proliferation of rabbit endometrium to a degree obtained with lesser amounts of intramuscular progesterone. Twenty-five mg doses of intravenous progesterone were capable of inducing an average progestational proliferation response in 6 animals to slightly above 1+. Five mg of intramuscular progesterone produced an average progestational proliferation response in the rabbit uterus of 2+ at 48 hours.

A final comparison of the efficacy of intravenous progesterone on rabbit endometrium utilizing the Corner-Allen progestational proliferation index is shown in Fig. 4. Here the effectiveness of intrauterine progesterone as compared to intravenous progesterone is shown. It was necessary to administer a saturation dose of 25 mg of intravenous progesterone to obtain the same progestational proliferation response to 1+ as found with 0.1 $\mu$g of intrauterine progesterone. It should be noted that maximum progestational proliferation of 4+ is rarely obtained with even larger doses of progesterone at 48 hours. Although the epithelial mitotic count is maximum at this point, the progestational proliferation response may require 72 to 96 hours for full development.

*Discussion.* Previously reported comparisons of relative efficacy of the several methods of progesterone administration have established that intramuscular administration of the hormone is approximately one thousand times(5,6) less effective in producing progestional proliferation than is intrauterine administration of progesterone. This marked difference in efficacy is the presumed result of inactivation of the hormone prior to its hematogenous transport to the endometrium. This biologic inactivation is delayed when the hormone is administered topically to the target tissue.

The current studies show that intravenous administration of progesterone is approximately twenty-five thousand times less effective than intrauterine administration of the hormone. Intravenous administration of the hormone is approximately 25 times less effective than the intramuscular route.

These comparisons establish the lessened biologic effectiveness of intravenous progesterone. Differences in effectiveness are usually the result of more rapid biologic inactivation either through absorption differences or circulation differences. Absorption of intravenously administered progesterone is instantaneous. Its lessened effectiveness is presumed to be the result of circulation of the hormone through the inactivating tissues prior to its arrival at the target tissue.

It appears that the previously reported rapid clearance of progesterone from the blood serum is not the result of tissue storage and continuing biologic effectiveness. It is the result of the rapid metabolism of the hormone and consequent lessened biologic effectiveness.

*Summary.* The biologic effectiveness of intravenously administered progesterone is approximately 25,000 times less than intrauterine administration of progesterone and 25 times less effective than the intramuscular route.

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# Secondary Rejection Phenomenon Elicited by Primary Homograft in Pretreated Rats. (24490)

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The graft rejection phenomenon has been considered explicable on an immunologic basis, in part due to the chronology of primary and secondary homograft rejections. Simonsen(1) has shown that prior immunization with skin from a kidney donor would sensitize the recipient so that accelerated kidney rejection would then occur. Dempster (2) showed that not only would skin grafting immunize against kidney from the same donor, but also that kidney grafts immunized against skin. Essentially the same findings were demonstrated by Billingham *et al.*(3) when it was shown that injections of an homogenate of lyophilized donor whole kidney would delay primary skin homograft rejection in pretreated neonatal rats. Based upon such reports indicating an antigenic relationship between skin and kidney, we evaluated a distinct anatomical component of the kidney in this relationship. The work to be reported here involved pretreating adult rats with rat glomerular basement membrane and subsequently performing an orthotopic skin homograft. In view of the report by Billingham *et al.*(4) concerning the role of nucleoproteins in graft rejection, RNA and DNA determinations were performed on our material.

*Methods.* A pool of several hundred rat kidneys was obtained from 5 different laboratories, all purchasing from different vendors. Glomeruli were isolated and basement membrane obtained from them by Greenspon's method(5) with the slight modification that no attempt at sterility procedures was attempted. Recipient and donor rats (of the skin grafts) were chosen from different lots so as to be as genetically disparate as is possible when purchasing from a single vendor. Two intraperitoneal injections of 40 mg (wet weight) of glomerular basement membrane in physiologic saline were given to 24 white, female, Holtzman strain rats (150 g) at an interval of one week. Seven days after the last injection, the orthotopic homograft was performed. One square centimeter of full-thickness skin was removed, trimmed of underlying fat and set into place on the prepared recipient area. The graft was secured with 8 to 10 stitches, and a pressure dressing of gauze was applied. Five days later dressing and stitches were removed. Numerous control autografts had previously validated the surgical technic. A group of 24 untreated homografted rats were given a second homograft from the same donor 2 weeks after rejection of the first graft.

RNA and DNA determinations on rat glomerular basement membrane were performed by the methods of Ceriott(6) and Webb(7) respectively. We are indebted to Dr. Max Rafelson, Dept. of Biochemistry for these determinations.

*Results.* Using rejection time criterion as complete breakdown of the graft, the base-line value of 13.2 ± 0.7 days was obtained for the primary homograft in the untreated rats. The pretreated rats rejected their primary homograft in about the same time, 7.3 ± 0.4 days, as the secondary homografts were rejected in the untreated rats, 7.8 ± 0.6 days. RNA and DNA values of 0.09 mg% and 0.05 mg% respectively were obtained.

*Discussion.* This preliminary report thus seems to substantiate the working hypothesis that there may be an immunologic relationship between a component of glomerular basement membrane and skin. This is not to imply that only the glomerular basement membrane is involved in the skin-kidney relationship. Currently other kidney structures are being investigated.

Aside from relating an anatomical component seemingly involved in the skin-kidney relationship, it would seem to us that greater significance may be attached to the fact that, as opposed to the works cited(1-3), the material employed for pretreatment was from a pooled source and not from the skin donors.

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The low nucleic acid content of our material limits coordination of our findings with the hypothesis presented by Billingham *et al.* (4). Experiments are underway to evaluate the role of a polysaccharide material isolated from rat glomerular basement membrane by a method reported elsewhere(8) in enhancing takes of skin grafts.

*Summary.* Intraperitoneal injections of pooled rat glomerular basement membrane preparations into rats subsequently homografted with a primary orthotopic skin graft facilitated a secondary rejection phenomenon on the part of such treated animals. The low nucleic acid content of the preparations limited correlation with the hypothesis of nucleoproteins as the transplantation antigens.

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## Occurrence and Enzymatic Degradation of Phosphoethanolamine.* (24491)

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The biological occurrence of phosphoethanolamine was first observed in bovine tumor tissue by Outhouse(1), who failed to find it present in normal tissues. Colowick and Cori(2) demonstrated the ester to be present in rabbit and pig intestine. Lately it has been shown to occur in a variety of normal tissues and often in quite large amounts(3,4,5,6). Thus far, the major biochemical interest of the compound lies in its possible role as an intermediary in the metabolism of phospholipids. Little is known of other possible functions, or of the enzymology of phosphoethanolamine metabolism. Roche and Bouchilloux (7) have shown that dog liver, human prostate, and beef kidney split phosphoethanolamine at pH 5.6, although at a lower rate than glycerophosphate. Strickland *et al.*(8) reported that alkaline phosphatase of various tissues readily split phosphoethanolamine, but that there was a relative inactivity toward this phosphate ester by acid phosphatase of these same tissues. This paper reports information about distribution of the compound in various tissues, and its enzymatic degradation by tissue homogenates under different conditions, *i.e.*, pH, buffer, metal ions, etc.

*Methods.* Phosphoethanolamine was synthesized by the method of Outhouse(9) and phosphate determinations were made by the method of Gomori(10). As soon as animals were sacrificed, samples of tissue were immediately homogenized with Teflon pestle in distilled water. Aliquots of the homogenate were treated with 10% trichloroacetic acid to remove proteins, and after removal of the acid with ether the resulting solution was concentrated *in vacuo.* Quantitative determination of phosphoethanolamine was then made by Dowex-50 chromatography as previously described(5). The incubation system contained in final volume of 11 ml, 9 ml of buffer (veronal-acetate, pH 4.5, or veronal, pH 9.0), 1 ml of substrate (15 x $10^{-5}$ moles of either phosphoethanolamine or beta-glycerophosphate, and 5 x $10^{-3}$ moles of $MgCl_2$), and 1 ml homogenate. Incubations were carried out in small Erlenmeyer flasks with shaking in

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TABLE I. Distribution of Phosphoethanolamine in Rat and Rabbit Tissues. Values expressed as mM/100 g dry tissue and probable error of the mean.

| Tissue | Rat* | Rabbit† |
|---|---|---|
| Liver | .18 ± .01 | 2.72 ± .06 |
| Kidney | .48 ± .07 | 2.34 ± .19 |
| Heart | .47 ± .17 | .36 ± .00 |
| Spleen | 2.24 ± .19 | 2.57 ± .30 |
| Brain | .50 ± .02 | .90 ± .00 |
| Muscle | .21 ± .05 | .10 ± .00 |
| Ret. cell sarcoma | 1.08 ± .06 | |
| Walker Ca. | .88 ± .04 | |

* Avg of 3 animals. † Avg of 2 animals.

constant temperature bath at 37°C. After one hour, the reaction was terminated by addition of 5 ml of 20% trichloroacetic acid. Inorganic phosphorous was determined similarly except that the substrate was omitted. A 1 ml aliquot of each homogenate was removed and kept at 100°C for 24 hrs for determination of dry weight. Values are expressed as $\mu$M of P released/g dry tissue/hr.

*Results.* Determinations made of phosphatase activity at intervals of 0.5 pH units from 4 through 10 showed the velocity of hydrolysis of phosphoethanolamine by various tissue homogenates to have 2 maxima at pH 4.5 and 9.0. Rates of hydrolysis by various tissue homogenates were compared in borate, veronal, tris, citrate, and acetate buffers. Although differences were slight, veronal at pH 9.0 and veronal-acetate at pH 4.5 gave maximum hydrolysis of the ester. The effect on hydrolysis rate of the ester at pH 9.0 by $Mg^{++}$ was determined on muscle, brain, and heart homogenates. The rate increased 100, 0, and 20% respectively, whereas $Ca^{++}$ at this pH had no effect. At pH 4.5, neither ion had any effect on hydrolysis of the ester by these 3 tissues; however $Mg^{++}$ increased hydrolysis of glycerophosphate by 50%. Under optimal conditions described, the reaction velocity using spleen and kidney homogenates at pH 9.0 remained essentially constant throughout a 120 minute period.

Table I illustrates concentration of phosphoethanolamine in 6 rat and rabbit tissues as well as 2 transplantable rat tumors. It can be seen there is a species difference, rabbit liver and kidney having considerably more of the ester than the corresponding tissues of the rat. The value for spleen in both species was large and similar results have been reported for lymphoid tissue by other authors(5,11).

Table II shows that for each tissue, glycerophosphate is hydrolysed by alkaline phosphatase at 2-6 times the rate for phosphoethanolamine. Only muscle of both species showed a lower ratio of approximately 1. The results of Table II also show that the ester of ethanolamine is hydrolysed at a considerably slower rate by acid phosphatase (pH 4.5) than glycerophosphate. It is obvious, however, that the ratio of hydrolysis of the 2 esters at the acid pH is not constant from one enzyme source to another, *viz.*, rat kidney, reticulum cell sarcoma, and Walker 256 carcinoma showed little activity toward the ester of ethanolamine; however the acid phosphatase of these 3 same tissues was quite active toward glycerophosphate. It is obvious from Table II that human prostate split glycerophosphate at 3 times the rate of phospho-

TABLE II. Hydrolysis of Phosphoethanolamine by Various Tissue Homogenates. Values expressed as $\mu$M/g dry tissue/hr.

| | Rat | | | | Rabbit | | | |
|---|---|---|---|---|---|---|---|---|
| | pH 4.5 | | pH 9.0 | | pH 4.5 | | pH 9.0 | |
| Tissue | PEt | BGP/PEt | PEt | BGP/PEt | PEt | BGP/PEt | PEt | BGP/PEt |
| Liver | 9 | 69 | 17 | 7.4 | 9 | 16 | 52 | 5.8 |
| Kidney | 1 | 1246 | 1800 | 3.3 | 41 | 6 | 480 | 4.5 |
| Heart | 6 | 24 | 160 | 3.5 | 1 | 78 | 19 | 2.0 |
| Spleen | 17 | 56 | 40 | 3.3 | 8 | 69 | 300 | 2.7 |
| Brain | 12 | 14 | 73 | 3.2 | 9 | 14 | 24 | 4.9 |
| Muscle | 12 | 15 | 18 | 1.3 | 23 | 7 | 23 | 1.0 |
| Reticulum sarc. | 3 | 75 | 57 | 4.3 | | | | |
| Walker 256 Ca. | 1 | 364 | 32 | 4.6 | | | | |
| Human prostate | 9760 | 3 | 38 | 4.0 | | | | |

BGP = Sodium beta-glycerophosphate. PEt = Phosphoethanolamine.

ethanolamine nevertheless this tissue contained the most active phosphatase for splitting of phosphoethanolamine of any tissues examined, irrespective of pH.

Rat liver homogenate gave rather low Q values with phosphoethanolamine; however these were approximately 7-fold when beta-glycerophosphate was the substrate. Further investigation showed that dialysis of liver homogenate increased hydrolysis of phosphoethanolamine and glycerophosphate 100 and 50% respectively. Furthermore, dilution of liver homogenate of 1:10 increased the Q value 4-fold; whereas similar dilution of kidney homogenate caused no change in the hydrolysis rate. Finally, it was found that boiled, undialysed liver extract inhibited hydrolysis of phosphoethanolamine by rat kidney homogenate at pH 9.0. Dialysis of liver extract removed this inhibition, but concentration of the dialysate followed by addition to kidney homogenate restored the inhibition.

*Discussion.* Comparison of Tables I and II indicates there is little correlation between amounts of free phosphoethanolamine in a given tissue as compared to amounts of enzyme which would split the compound, *i.e.*, rat kidney and brain had approximately the same amounts of free ester, *viz.*, (.48 *vs.* .50); however kidney was about 25 times as active as brain at pH 9.0 in hydrolysing phosphate from the compound.

Our findings shown in Table II are in agreement with those of Roche and Bouchilloux (7) who had shown that phosphoethanolamine is split at an acid pH although at a lower rate than glycerophosphate. Our results are at variance with Strickland *et al.*(8) in that we found rat and rabbit brain homogenate to be fully as active under acid conditions toward phosphoethanolamine as most other tissues of these species. We also differ in that we found human prostate to contain the most active phosphatase of any tissue examined; whereas Strickland *et al.*(8) reported almost complete inactivity of this tissue toward phosphoethanolamine under acid conditions.

The increased Q value obtained with dilution and dialysis of liver homogenate suggests that the activity of this tissue phosphatase toward phosphoethanolamine might be obscured by a transphosphorylation reaction. Suitable aliquots of the remaining hydrolysate were chromatogramed on paper and showed equal quantities of ethanolamine and inorganic phosphate released with corresponding disappearance of a similar amount of phosphoethanolamine. Schmidt *et al.*(12) have shown that intestinal phosphatase is inhibited by phosphate ion. However, addition of .0075 mM phosphate, an amount equivalent to that found in 1 ml of liver homogenate, had no effect on activity of liver phosphatase on phosphoethanolamine. These results indicate that rather than a transphosphorylation, or inhibition by phosphate ion, there is perhaps a competition for the phosphatase between phosphoethanolamine and other phosphate esters present in liver.

*Summary.* 1. Optimum conditions for hydrolysis of phosphoethanolamine by tissue homogenates have been determined to be: veronal-acetate, pH 4.5, and veronal, pH 9.0, $Mg^{++}$, 5 x $10^{-3}$ M. 2. Concentration of phosphoethanolamine was determined in 6 rat and rabbit tissues as well as 2 transplantable tumors. 3. Alkaline phosphatase of the various extracts readily hydrolysed the ester and at greater rates than acid phosphatase. 4. Phosphoethanolamine was more readily hydrolysed by acid phosphatase of human prostate than of any other tissue examined. 5. Dilution and dialysis experiments with liver indicate there is a competition for phosphatase between phosphoethanolamine and other phosphate esters present in liver.

The authors are indebted to Mr. Said Nehorayan and Miss Verna Lee Nitteberg for technical assistance.

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## Effect of Properdin on Whole Body Irradiated Mice and Rats.* (24492)

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The parenteral administration of properdin of heterologous origin into rats and mice has been reported to protect significantly against $LD_{100}/30$ day and $LD_{100}/6$ day doses of whole body x-irradiation(1,2). Degree of protection was reported to be dependent on dose, route, and time of administration of properdin. Ross(2) observed that mice exposed to 600 r and treated with 50 units of partially purified bovine properdin administered intravenously showed a 4-fold greater 30 day survival rate than the untreated controls. However, mice treated with properdin on the second, third, fourth, seventh, or tenth postirradiation days showed similar or accelerated death rates as compared to the controls. In order to further assess the role of properdin in protection of animals against the effects of whole body x-irradiation, experiments were conducted to determine the value of properdin as a therapeutic agent.

*Materials and methods.* Adult albino mice and adult albino rats (Sprague-Dawley) were randomly arranged into groups of varying numbers according to the design of each experiment. Dosage of x-rays administered for each experiment is given in the section on results. The x-irradiation was administered from above the animals by means of a Westinghouse Quadrocondex x-ray machine. The technical factors were: 250 KVP; 15 ma, 1 mm aluminum and 0.5 mm copper filters in addition to inherent filtration of 2.5 mm aluminimum and 0.25 mm copper; size of field, whole-body; target distance, 16 inches. The mice were irradiated in groups of 10 in a cylindrical container having a diameter of 18.5 cm and a height of 3 cm. The rats were irradiated in groups of 4 in a cylindrical container having a diameter of 23 cm and a height of 5.5 cm. Average dose rate as measured with a Victoreen r meter was approximately 90r per minute when recorded in the center of a lifelike wax phantom. Variation in radiation dose did not exceed 2r per minute when measured in any position in the container. Properdin solutions were prepared from fresh bovine serum according to the zymosan adsorption technic of Pillemer *et al.*(3) and also by a modified Cohn ethanol fractionation procedure. In addition, purified human properdin was used in the experiments.† The titers of the partially purified properdin (PPP) solutions and the human properdin (HP) were determined by the zymosan assay technic described by Pillemer *et al.*(3).‡ Route and time of administration of the properdin solutions is given in each experimental design.

*Results.* In the first experiment, 5 randomly selected groups of mice were exposed to a single 550 r total body dose of x-irradiation. Animals of the first group of 11 mice were in-

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† We are indebted to Dr. B. E. Sanders of Merck Inst. of Therapeutic Research for purified human properdin.

‡ Original RP and R3 assay reagents were kindly supplied by the late Dr. Louis Pillemer and Mr. Earl W. Todd of the Inst. of Pathology, Western Reserve Univ., Cleveland, O. Portions of reagents were used to standardize subsequent assay reagents prepared in this laboratory.

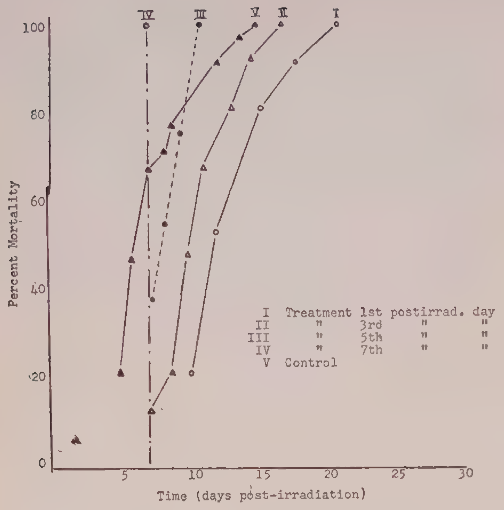

FIG. 1. Mortality of adult albino mice treated intrav. with 50 units of partially purified bovine properdin (PPP) following 550 r total body x-irradiation.

jected intravenously with 50 units of PPP on the first postirradiation day. The next 3 groups of 11 mice each were treated with 50 units of intravenously administered PPP on the third, fifth and seventh postirradiation days, respectively. The fifth group of 40 mice was used as untreated controls. The mortality curves following this irradiation and treatment procedure are shown in Fig. 1. It can be seen that the intravenous injection of PPP did not afford protection to the x-irradiated mice regardless of time of administration. However, there appeared to be a trend toward delayed mortality if treatment was initiated early following total body x-irradiation.

In another experiment, the first group of 10 mice each received 50 units of HP intravenously on first postirradiation day following a single exposure to 550 r of total body x-irradiation. A similar number of mice received the same treatment on third postirradiation day and a third group of 10 mice each received 50 units of HP intravenously on fifth postirradiation day. A fourth group of 33 mice were used as untreated controls. The mortality ratios are shown in Table I. It can be seen that postirradiation treatment with 50 units of HP administered intravenously did not yield protection to the whole body x-irradiated mice regardless of time of administration.

TABLE I. Mortality of Adult Albino Mice Treated Intravenously with 50 Units of Purified Human Properdin (HP) following 550 r Total Body X-irradiation.

| Treatment | Treatment day (PID)* | No. of mice | Final mortality % | Final mortality Day |
|---|---|---|---|---|
| HP | 1 | 10 | 100 | 12 |
| " | 3 | 10 | " | 9 |
| " | 5 | 10 | " | 9 |
| Control | | 33 | " | 15 |

* PID, postirradiation day.

It is difficult to obtain properdin with high activity, that is, high unitage/unit volume. To increase host systemic concentration of properdin, it was suggested that intraperitoneal injection of large doses of properdin solution would enable the host to absorb more properdin than could be administered by intravenous route. This increased level of properdin might then be effective in protecting against the effects of whole body x-irradiation. To test this hypothesis, mice were subjected to 600 r of whole body x-irradiation and randomly separated into 5 groups. The first group of 20 mice received 200 units of PPP intraperitoneally on the third post-irradiation day; the second and third groups of mice (20 mice/group) each received 200 units of PPP injected intraperitoneally on fifth and seventh postirradiation days respectively. The fourth group of 20 mice received a total of 600 units of PPP administered intraperitoneally at a dosage of 200 units/day given on third, fourth and fifth postirradiation days. The 25 mice of fifth group were held as controls. The mortality curves of this irradiation and treatment experiment are shown in Fig. 2. The results were that properdin was not effective as a therapeutic agent against x-irradiation even in concentrations as high as 600 units when given by intraperitoneal route, regardless of time of administration.

Various investigators have reported that mouse serum is deficient in complement components(4-6). The lack of bactericidal activity of mouse serum reported by Marcus, *et al.*(7) could be explained as a result of deficient complement components. Since the properdin system has been reported by Pille-

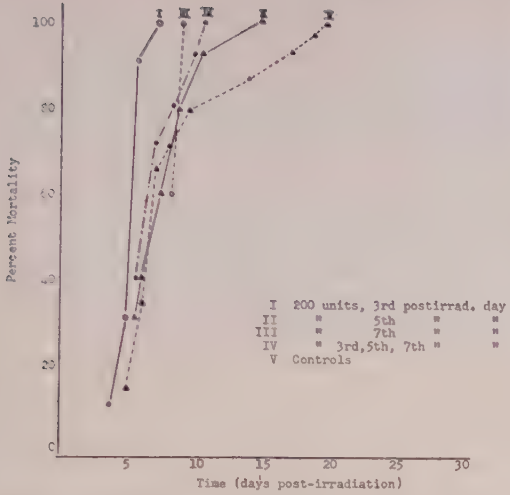

FIG. 2. Mortality of adult albino mice treated intraper. with partially purified bovine properdin (PPP) following 600 r total body x-irradiation.

mer *et al.*,(8) to require the 4 components of complement and $Mg^{++}$ for its activation, failure of properdin to protect mice against effects of whole body x-irradiation might be explained on this basis. An experiment was carried out to determine if the properdin system could be activated *in vivo* in the mouse by concomitant injection of guinea pig complement with properdin. Groups of mice were subjected to 500 r of total body x-irradiation and treated with 200 units of PPP and 33 units of guinea pig complement (GPC′). The mixture was administered intraperitoneally rather than intravenously since a total volume of 1.1 ml was injected. The results are shown in Table II. The x-irradiated mice were not protected even though all components of complement were present. Mortality rates of treated mice were, in all cases, accelerated as compared to mortality rates of the control group. GPC′ was not toxic in the concentrations employed.

The hypothesis was proposed that mouse serum inactivates guinea pig complement *in vivo*. The failure of properdin to protect x-irradiated mice might be explained on this basis. It was reported that x-irradiation did not affect rabbit complement titers(9,10), and Ross(2) reported that complement component titers of the rat are not affected by x-irradiation. In other experiments, therefore, rats were used.

TABLE II. Mortality of Adult Albino Mice Treated Intraperitoneally with 200 Units of Partially Purified Bovine Properdin (PPP) and 33 Units of Guinea Pig Complement (GPC′) following 550 r Total Body X-irradiation.

| Treatment | Treatment day (PID)* | No. of mice | Final mortality % | Final mortality Day (PID) |
|---|---|---|---|---|
| PPP + GPC′ | 1 | 10 | 100 | 8 |
| GPC′ | 1 | 5 | " | 14 |
| PPP + GPC′ | 3 | 10 | " | 9 |
| GPC′ | 3 | 5 | 80 | 30 |
| PPP + GPC′ | 5 | 10 | 100 | 10 |
| GPC′ | 5 | 5 | " | 9 |
| PPP + GPC′ | 7 | 10 | " | 8 |
| GPC′ | 7 | 5 | " | 10 |
| Control | | 20 | " | 13 |

* PID, postirradiation day.

Groups of rats were subjected to 720 r of whole body x-irradiation and randomly divided into 7 groups. The first group of 15 rats received 200 units of PPP injected intraperitoneally on the third postirradiation day. The next two groups of 15 rats per group each received 200 units of PPP intraperitoneally on the fifth and seventh postirradiation days, respectively. The fourth group of 15 rats received a total of 1000 units of PPP intraperitoneally at a dosage rate of 200 units/day for 5 consecutive days starting with third postirradiation day. The fifth group of 15 rats received a total of 500 units of PPP administered intraperitoneally at 100 units/day for 5 consecutive days starting with third postirradiation day. The sixth group of 14 rats received a total of 250 units of PPP intraperitoneally injected at 50 units/day for 5 consecutive days commencing with third postirradiation day. The seventh group of 20 rats was untreated and held as controls. The results of this experiment are shown in Table III. It can be seen that properdin did not protect rats from the effects of whole body x-irradiation, even in doses as high as 1000 units when administered by intraperitoneal route. Mortality rates of groups receiving multiple injections were observed to be similar to or slightly accelerated compared to the control group and groups receiving a single treatment. It is also observed that a trend toward delayed mortality appears to occur if a single injection of properdin is given to rats early in treatment following whole body x-irradiation.

TABLE III. Mortality of Adult Albino Rats Treated Intraperitoneally with Partially Purified Bovine Properdin (PPP) following 720 r Total Body X-irradiation.

| Treatment PPP (units) | Treatment day (PID)* | No. of rats | Final mortality |  |
|---|---|---|---|---|
|  |  |  | % | Day |
| 200 | 3 | 15 | 87 | 30 |
| 200 | 5 | ” | 100 | 11 |
| 200 | 7 | ” | ” | 11 |
| 200 | 3, 4, 5, 6, 7 | ” | ” | 12 |
| 100 | ” | ” | ” | 11 |
| 50 | ” | 14 | ” | 11 |
| Control |  | 20 | ” | 15 |

* PID, postirradiation day.

*Discussion.* It has been reported by Ross (2) that properdin levels in rats and mice decrease to undetectable levels following whole body x-irradiation. Linder(11) reported that properdin levels of 500r x-irradiated rats falls to 52% of normal on third postirradiation day and reaches a minimum of 34% of normal by eighth postirradiation day. The properdin level begins to rise and reaches 60% of normal on thirteenth postirradiation day. We observed that properdin levels of 720r x-irradiated rats fell rapidly and reached undetectable levels between fifth and seventh postirradiation days. Furthermore, properdin levels of 500r x-irradiated mice rapidly decreased to 10% of normal levels by fifth postirradiation day and remained undetectable until all animals died on fifteenth postirradiation day.

If properdin has therapeutic value for whole body x-irradiated animals, it seems apparent that treatment should be given at a time when the host endogenous levels are at a minimum. In all our experiments, the time of administration of exogenous properdin was varied to include postirradiation time during which some endogenous properdin is still present (first postirradiation day) to when undetectable levels are found (fifth to seventh postirradiation days). It was found that properdin did not afford any protection to the whole body x-irradiated animals in doses as high as 600 units given intraperitoneally for mice and 1000 units by this route for rats. It was observed, however, that time of administration of exogenous properdin appears to be an important factor. Trends toward delayed mortality were observed to occur if treatment was initiated early following whole body x-irradiation. In all cases, treatment on the seventh postirradiation day accelerated the death rate considerably as compared to the controls. This observation was also reported by Ross (2).

It has been difficult to obtain properdin solutions of high activity/unit volume. Consequently, intravenous injections of properdin of high activity could not be completed without introducing large fluid volumes. The choice of intraperitoneal route of injection was made on the assumption that properdin could be absorbed by the animal in doses greater than could be administered intravenously without causing physiological stress. It is of interest to note that in the bactericidal tests conducted with the sera of x-irradiated rats, no bactericidal activity was measurable when the properdin levels of the animals were undetectable. Perhaps failure of properdin to protect the animals against bacteremia that follows whole body x-irradiation might be explained on the asumption that sufficient levels of systemic properdin were not obtained. However, *in vitro* studies by Wardlaw and Pillemer(12,13) show that bactericidal activity of the properdin system occurs with a system containing as little as 0.01 units of properdin. It is recognized that extrapolation of *in vitro* observations to *in vivo* predictions must be guarded. They also reported that concentrations of properdin above 10 units was inhibitory in the *in vitro* bactericidal process. Possibly a minimum of properdin would give maximal *in vivo* protection. In an initial experiment in this laboratory, use of properdin of low activity (1 unit/ml) yielded 60% 30 day mortality among 500r x-irradiated mice compared to 100% mortality of the controls in 15 days. However, attempts to confirm this observation have been uniformly negative.

*Summary.* Under conditions employed postirradiation treatment of mice and rats with partially purified bovine properdin and purified human properdin administered intraperitoneally or intravenously did not afford protection against the effects of whole body x-irradiation. There appeared to be a relationship between delayed mortality and time of injection.

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## Enzymatic Determination of Sorbitol in Animal Tissues.* (24493)

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Chemical methods for determination of sorbitol and related polyols depend upon degradation of these substances by ferricyanide(1, 2) or periodate(3,4). These procedures suffer from the disadvantage of extreme lack of specificity, since a wide variety of sugars other than acyclic polyols react with these reagents. Although interference by substances such as glucose can be minimized by pretreatment with fermenting yeast(3) or by examining the reactions under carefully controlled conditions (4), application of these methods to determination of polyols in tissue extracts is not altogether satisfactory. Purification of a soluble liver polyol dehydrogenase(5) which catalyzes the reversible oxidation of certain 5, 6 and 7 carbon polyols to ketoses(6,7) made possible the present method for enzymatic estimation of sorbitol. As little as 1 $\mu$g of sorbitol can be measured by rapid and simple spectrophotometric procedures, not subject to interference by glucose or other naturally occurring sugars.

*Methods and materials.* Spectrophotometric measurements were made with Beckman model D.U. spectrophotometer using cells of 1 cm light path. Most sugars and polyols used were obtained from Pfanstiehl Laboratories, Waukegan, Ill., and were of the highest grade of purity. L-Iditol(8) was donated by Dr. Jean Sice. DPN and TPN were more than 80% pure when assayed enzymatically (9,10). DPNH was prepared by either chemical(11) or enzymatic(12) reduction of DPN. Ketoses were estimated by the methods of Dische and Borenfreund(13) or Roe (14). Protein was determined spectrophotometrically(15). Molar extinction coefficient of pure DPNH at 340 $\mu$ was assumed to be 6220.

*Results. Purification of soluble polyol dehydrogenase of liver. Assay.* Activity of the enzyme at each stage of purification was determined in a reaction mixture containing 0.033 M sodium pyrophosphate buffer pH 9.1, 0.0006 M DPN and 0.033 M sorbitol. Total volume was 1.5 cc. The reaction was initiated by addition of enzyme in total volume of 0.1 cc or less. Absorbency at 340 $\mu$ was measured at 30 second intervals at 25°. Reaction velocities were determined from zero order portions of plots of change in absorbency *vs.* time. One unit of activity was defined as equal

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to change in absorbency of 0.001/minute. Specific activities were defined as units/mg protein. Enzyme activity was proportional to amount of protein added over a wide range of concentration.

*Initial extract.* This was based on the procedure of Blakley(6). Either fresh or frozen rat liver was chopped with scissors and washed many times with cold tap water. After washing with deionized water the tissue was homogenized in Waring blendor with 4 volumes of 0.01 M sodium phosphate buffer pH 7.8 at 2°. The homogenate was centrifuged at 3,000 x g for 20 minutes and the precipitate discarded. In some experiments the pH of initial extract was adjusted to 4.7 by addition of 10 N HCl at 2°, and the precipitate which formed was discarded. The supernatant fluid was adjusted to pH 7.4 with 5 N NaOH and a further precipitate removed. The supernatant fluid resulting from either type of treatment was then treated with chloroform (0.25 vol) and ethanol (0.25 vol) according to Blakley(6). The aqueous extracts resulting from this treatment were dialyzed against deionized water at 2° until free of organic solvents. Any insoluble material remaining after dialysis was removed.

*Ammonium sulfate fractionation.* Solid ammonium sulfate was added slowly at 2° until the saturation was 0.4. The mixture was stirred one hour and the precipitate removed. Ammonium sulfate was added to the supernatant fluid until saturation had been raised to 0.7. Incomplete recoveries of enzyme were obtained if the saturation with ammonium sulfate was raised to only 0.6 as stated elsewhere(5). The precipitate was harvested and dialyzed against deionized water until free of ammonium sulfate. Insoluble material remaining after dialysis was removed.

*Ethanol fractionation.* The solution was cooled to 0° and ethanol (–25°) was added dropwise until a final concentration of 20% was reached. After addition of all of the ethanol, the mixture was stirred for 10 minutes and centrifuged. In a similar manner the precipitates resulting from addition of ethanol to final concentrations of 30, 40 and 50% were collected. The ethanol fractions were suspended in and dialyzed against deionized water at 2°; any insoluble material remaining after dialysis was removed. The material possessing the highest specific activity for sorbitol oxidation was in the 40-50% ethanol fraction. However, this was only the case if the ethanol fractionation was carried out as described above. If ethanol was added in one step to a final concentration of 40%, a great deal of polyol dehydrogenase coprecipitated with a large bulk of inert protein.

*Properties of purified enzyme.* The purified preparations exhibited specific activities for sorbitol oxidation ranging from 1600 to 2500 units/mg protein. At a final concentration of 0.033 M, L-iditol, ribitol and xylitol were oxidized at 96, 83 and 56% of the rate of sorbitol. TPN was reduced by sorbitol at less than 2% of the rate with DPN. The enzyme was stable for some months when stored at –20°, but repeated freezing and thawing was deleterious. In the standard assay system with sorbitol omitted, and containing 100 units of enzyme, DPN was not reduced by glucose (0.05 M), glycerol (0.02 M) n-propanol (0.02 M), m-inositol (0.01 M) and ethanol (0.02 M). At pH 7.4 in phosphate buffer, DPNH was oxidized by D-fructose (0.03 M) and L-sorbose (0.03 M) was oxidized at 86% the rate of fructose. DPNH was not oxidized upon the addition of DL-glyceraldehyde, dihydroxyacetone, monohydroxyacetone or potassium pyruvate (0.01 M).

*Equilibria.* Others(6,7) have shown that the soluble polyol dehydrogenase of mammalian liver catalyzes, *inter alia*, the following reactions:

$$
\begin{array}{llllll}
\text{Sorbitol} + & \text{DPN}^+ & \rightleftarrows \text{D-Fructose} + & \text{DPNH} + \text{H}^+ & & (1)\\
\text{L-Iditol} + & \text{"} & \rightleftarrows \text{L-Sorbose} + & \textit{Idem} & & (2)\\
\text{Ribitol} + & \text{"} & \rightleftarrows \text{D-Ribulose} + & \text{"} & & (3)\\
\text{Xylitol} + & \text{"} & \rightleftarrows \text{L-Xylulose} + & \text{"} & & (4)
\end{array}
$$

The equilibrium constant K = [Ketose] [DPNH]/[Polyol][DPNH] for reaction (1) was found by Blakely(6) to be 0.24 at pH 8.0 and 20°. The equilibrium of other pyridine nucleotide linked alcohol dehydrogenases is displaced to the right with rise in pH, since a proton is liberated when the coenzyme is reduced(9,17). The equilibrium for sorbitol oxidation by the purified enzyme was determined at various pH values. The vessels contained 200 units of enzyme/1 cc of reaction

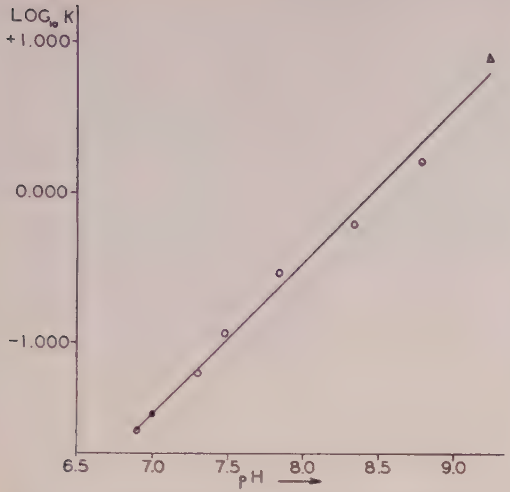

FIG. 1. Variation of equilibrium constant for transformation of sorbitol to fructose with pH. ○, Tris buffer; ●, phosphate buffer; △, pyrosphosphate buffer.

mixture. and initial concentration of DPN was 0.00016 M. At each pH, mixtures of sorbitol and D-fructose were added at approximately 100 to 300 times the molar concentration of DPN. Equilibrium was considered to be established when no further change in absorbency at 340 m$\mu$ occurred within 30 minutes. Three different ratios of sorbitol to fructose were employed for determination of K at each pH value. A plot of $\log_{10}K$ against pH yielded a straight line of unit slope (Fig. 1). The thermodynamic equilibrium constant $K_H = K \times [H^+]$ was estimated graphically to be $3.35 \times 10^{-9}$ for reaction (1) at 25°. This is in good agreement with the value of K at pH 8.0 reported by Blakley(6) and with a previously reported value(5) of $2.09 \times 10^{-9}$ obtained from determinations made at a smaller number of pH values. The value of K for reaction (3) was calculated from amount of DPNH formed in the presence of limiting concentrations of ribitol and excess DPN, on the assumption that 1 mole of D-ribulose was formed/mole of DPN oxidized. In sodium pyrosphosphate buffer of pH 9.0 an average value of K = 0.107 was found. The value of K for the xylitol ↔ D-xylulose reaction (equation 4) was not determined. However, it was observed that at pH 9, in the presence of 0.0005 M DPN, addition of 0.05 $\mu$mole of xylitol/1 cc of reaction mixture led to formation of 89% of the theoretical amount of DPNH, which would be expected if oxidation of xylitol had gone to completion. It is clear that there is a large difference between equilibrium constants for reactions 3 and 4.

*Determination of sorbitol.* From the value of $K_H$ for reaction (1) it may be calculated that at pH 9 in a final volume of 2 cc, containing initially 1 $\mu$mole of DPN, the conversion of 0.2 $\mu$mole of sorbitol to D-fructose is more than 98% complete. Thus it would be expected that addition of sorbitol in quantities of 0.2 $\mu$mole or less would result in formation of stoichiometric amounts of DPNH. This was found to be the case. In the presence of 0.1 $\mu$mole of added sorbitol, further addition of 0.1 $\mu$mole of D-fructose did not alter detectably the final change in absorbency, as would be expected from the value of $K_H$. Addition of D-fructose in molar concentrations 100 times that of sorbitol reduced the final change in absorbency to about one-half of the theoretical. The following procedure was adopted to determine sorbitol in tissue extracts. The tissue was deproteinized by 1 of 2 methods. The first of these involved homogenization of tissue with 9 volumes of 5% TCA. The homogenate was centrifuged, the residue washed with TCA, and the combined acid soluble extract used. The second method of deproteinization was effected by homogenizing with 9 volumes of 66% ethanol. The homogenate was centrifuged and the precipitate washed with 66% ethanol. The combined ethanol extracts were stripped of ethanol under reduced pressure, and the aqueous residue extracted 3 times with chloroform. Traces of chloroform were removed from the aqueous extract by aeration. The deproteinized extracts were then de-ionized by passage over columns of the anion-cation strong base, strong acid ion exchange resin Amberlite MB-1 (Rohm and Haas) until free of ionic material as judged by conductivity determinations. The de-ionized extracts were concentrated under reduced pressure. At this stage, total polyol present was determined, using a suitable aliquot in final volume of 2 cc containing 100 $\mu$mole of sodium pyrosphosphate buffer of pH 9.1, 1 $\mu$mole DPN and sufficient amounts of purified liver enzyme to allow the system to come into equilibrium within 20 minutes. An aliquot of de-ionized extract was also subjected to cysteine-carbazole reaction of Dische and Borenfreund(13) to determine level of ketoses present. Aliquots of de-ionized extracts were also chromatographed on Whatman number 42 filter paper in an ascending system at 25° using 85% ethanol as solvent. Standards of sorbitol (100 $\mu$g) and ribitol (100 $\mu$g) were run alongside these samples. The following values for $R_{sorbitol}$ (movement of substance/movement of sorbitol) were observed in this system: L-iditol, 0.94; D-mannitol, 1.00; ribitol, 1.20; glycerol, 1.42; m-inositol, 0.23; D-glucose, 0.98 and D-fructose, 0.98. Location of the carbohydrates on the chromatograms was determined by spraying with ammoniacal silver nitrate. In each determination, 2 chromatograms were run side by side. One of these was sprayed to determine the position of carbohydrates present, while the other was cut into strips 1 cm in length. Each strip was eluted with a small volume (3 cc or less) of water, and a suitable aliquot of the eluate was used for determination of polyol present by the enzymatic method.

Recoveries of greater than 92% were found if a solution of pure sorbitol was treated with TCA, and subjected to de-ionization and paper chromatographic procedures here described. Sorbitol could not be detected in either rat liver or in heparinized rat blood (adult males weighing between 350 and 400 g), under conditions where 2 mg of sorbitol/100 g of tissue could have been detected. It may be added that after de-ionization of the deproteinized extracts of these tissues, amount of ketose present was insignificant, and incapable of leading to errors in determination of sorbitol due to alteration of the equilibrium of reaction(1) by D-fructose. Sorbitol added to deproteinized, heparinized rat blood was also recovered to the same extent.

Similarly, no difficulties were encountered in estimation of sorbitol added to rat urine. It must be emphasized that the de-ionization procedure employed precluded interference by ionic substrates which would form DPNH by action of DPN-linked dehydrogenases which might be present in the purified liver enzyme used. Failure of glucose, glycerol, ethanol and inositol to reduce DPN in the presence of liver ketose reductase also excluded interference by these substances. Paper chromatography of de-ionized extracts of rat liver showed the presence of material which reacted with ammoniacal silver nitrate and migrated to the same positions as glycerol, glucose and m-inositol.

In 2 separate experiments it was found that the coagulating gland of sexually mature rats contained an hexitol in amounts equivalent to 25 and 37 $\mu$moles/100 g of fresh tissue, and which migrated on paper to the same position of sorbitol. Work from this laboratory showed that fructose-secreting accessory sexual tissues of the rat and guinea pig catalyzed the transformation of sorbitol to fructose readily(5,18). The finding of an hexitol resembling sorbitol in the coagulating gland of the rat, which secretes large amounts of fructose, suggests that sorbitol may be an intermediate in biosynthesis of fructose by this tissue. This contention is in full accord with studies of Hers(19,20) on seminal vesicle of the sheep. The latter tissue, which also secretes fructose, contained sorbitol in its secretion, and also enzymes which reduce glucose to sorbitol and oxidize sorbitol to fructose.

*Summary.* An enzymatic method for determination of sorbitol is described. The method is based upon reduction of DPN by sorbitol catalyzed by a purified preparation of soluble ketose reductase of rat liver. No polyol which reacts with this enzyme could be detected in blood or liver of the rat, whereas considerable amounts of an hexitol resembling sorbitol were found in the coagulating gland.

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## Chemical Studies of Mucopolysaccharides of Rat Blood Platelets. (24494)

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Isolation of a sulfated mucopolysaccharide from blood platelets of rats has been reported (1). Subsequent investigation on larger quantities of extracts of rat platelets (200 to 500 μg) revealed another mucopolysaccharide-rich fraction that is not sulfated. This report describes the separation and further chemical characterization of these two fractions by paper electrophoresis, hydrolysis, and paper chromatography.

*Materials and methods.* Male Sprague-Dawley rats weighing 272-477 g were used as platelet donors. Platelets labeled *in vivo* with $Na_2S^{35}O_4$ and harvested by the procedure described(1) were lyophilized or frozen for storage. Mucopolysaccharides (MPS) were extracted from platelets by alkali treatment(1) or by trypsin digestion. The trypsin digestion medium was made by adding 0.1 ml of a 0.1% trypsin[†] solution to 0.9 ml of M/10 $NaHCO_3$ buffer (pH 8.3, M/1000 with respect to $CaCl_2$). One milliliter of this mixture was added for each 13.3 x $10^9$ platelets and reacted for 1 week at 34°-38°C. The undissolved material was removed by centrifugation at 2190 x g for about 30 minutes. The supernatant was neutralized with 1 N HCl, diluted to 10 ml with water, and repeatedly treated with chloroform-amyl alcohol reagent (2) to remove protein. The resulting supernatant was dialyzed and lyophilized(1). In addition to the method of electrophoresis used before [essentially that of Rienits(3)], we also used as the electrolyte a 35% solution of propanol in M/15 phosphate buffer (pH 6.4) a solvent system similar to that used by Kerby(4) for chromatographic separation of heparin and chondroitin sulfate(CSA). The extract was electrophoresed on strips of Whatman #2 filter paper (3.0 x 30.5 cm) for 5 hours at 10.8 V/cm. The two MPS-rich fractions were located on paper strips by staining with Alcian blue. Each MPS-rich fraction was eluted from unstained paper electrophoresis strips run by the Rienits procedure, dialyzed, lyophilized, and hydrolyzed by modification of the method of Glegg and Eidinger (5). Two hundred to 500 μg (estimated by comparing staining density of small samples on paper strips with that of known amounts of CSA) of each fraction and 12 mg of Dowex 50 cation exchange resin[‡] in the hydrogen form and 0.1 ml of water were heated at 100°C for 33 or 40 hours in a sealed tube with a volume of about 3 ml. In this procedure hexosamines released during hydrolysis adsorb to the resin, and sugars and uronic acids remain in solution. The hydrolysis was carried out on 2 different batches of each fraction as well as in control samples of CSA,§ of paper electrophoresis strips, and of resin alone. Sugars and uronic acids were chromatographed on Whatman #1 filter paper. Each paper was stapled together to form a cylinder and placed in glass jar with one of the following solvents: (1) butanol-acetic acid-water (4:1:5), bottom layer used to saturate the atmosphere; (2) phenol-water (71:29); (3) butanol-pyrodine-water (5:3:2) of Yosizawa (6); (4) pyridine-ethyl acetate-acetic acid-water (5:5:1:3), and (5) pyridine-ethyl acetate-water (11:40:6) used to saturate the atmosphere(7). The solvent was allowed to rise to the top of the paper 3 times. Material from fraction 1 (faster moving fraction) was chromatographed in all 4 systems, but fraction 2 material was run only in the last 2. The papers were sprayed with aniline hydrogen phthalate to reveal sugars and uronic acids. Hexosamines were eluted from the resin with 0.5 N HCl and chromatographed in water-saturated collidine by Woodin's procedure (8). These papers were sprayed either with ninhydrin or with the Elson-Morgan reagent (9) to reveal hexosamines. Once when ninhydrin was used, the pentose sugars formed as oxidation products of hexosamines(10) were eluted and chromatographed in the pyridine-ethyl acetate-acetic acid-water system described to confirm identification of the hexosamines. Hydrolysis was carried out on 2 different batches of each fraction as well as on control samples of CSA, of paper electrophoresis strips, and of resin alone.

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* Operated by Union Carbide Corp. for U. S. Atomic Energy Comm.

[†] The trypsin was a crystallized, lyophilized, salt-free preparation (TL 550) obtained from Worthington Biochemical Sales Co.

[‡] Dowex-50 resin of analytical grade, 50 to 100 mesh, obtained from Bio-Rad Laboratories.

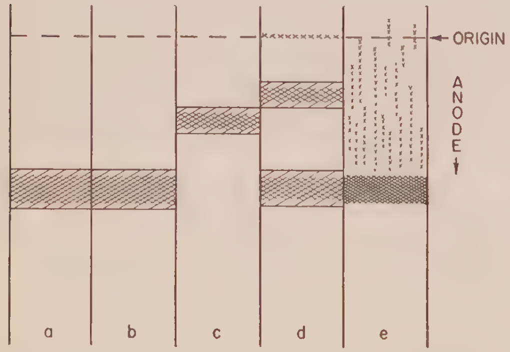

FIG. 1. Paper electrophoresis of mucopolysaccharides and platelet extract by modified Rienits procedure (conditions: M/10 phosphate buffer, pH 6.6; 3 hr; 5°C; 10.8 V/cm). a, chondroitin sulfate; b, heparin; c, hyaluronic acid; d, platelet extract; e, autoradiogram of d.

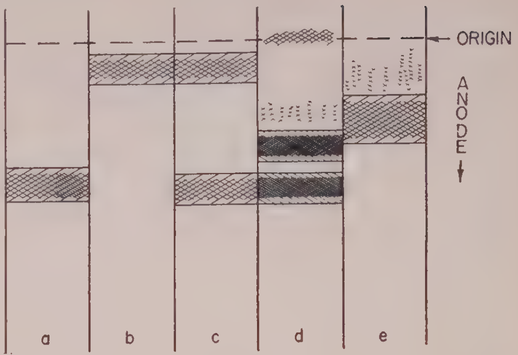

FIG. 2. Paper electrophoresis of mucopolysaccharides and platelet extract in propanol-phosphate buffer system (conditions: 35% propanol, 65% M/15 phosphate buffer, pH 6.4; 5 hr; 5°C; 10.8 V/cm). a, chondroitin sulfate; b, heparin; c, mixture of chondroitin sulfate and heparin; d, platelet extract; e, hyaluronic acid.

*Results.* Two fractions that stained with Alcian blue were observed on paper electrophoresis strips that were run either in phosphate buffer or in 35% propanol-phosphate buffer (Figs. 1 and 2). In both systems fraction 1 had the same electrophoretic mobility as CSA. Autoradiograms of paper electrophoresis strips of extracts of $S^{35}O_4^{--}$-labeled platelets showed that fraction 1 was sulfated (Fig. 1). In the phosphate buffer system, fraction 2 (the slower moving fraction) moved slightly behind hyaluronic acid (HA)‖ but overlapped it (Fig. 1), whereas in the 35% propanol-phosphate buffer that separates CSA and heparin,¶ fraction 2 moved in front of and overlapped the position of HA (Fig. 2). Neither fraction had a mobility corresponding to that of heparin.

Chromatograms of the hexosamines sprayed either with ninhydrin or with Elson-Morgan reagent showed the presence of galactosamine in fraction 1, whereas fraction 2 contained glucosamine. When the hexosamine spots that were oxidized with ninhydrin were eluted and

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§ The chondroitin sulfate was obtained from Nutritional Biochemicals Corp.

‖ The HA from vitreous humor was obtained from Mann Research Labs.

¶ The heparin was Liquaemin Sodium from Organon.

TABLE I. Chromatographic Detection of Sugars and Uronic Acids in Mucopolysaccharides.

| | Fraction 1 | | | Fraction 2 | | | CSA | | | Resin blank | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Amt* | Frequency† | Solvent systems | Amt | Frequency | Solvent systems | Amt | Frequency | Solvent systems | Amt | Frequency | Solvent systems |
| Uronic acid | ++ | 5/5 | 4 | — | 0/2 | 2 | ++ | 7/10 | 4 | — | 0/6 | 2 |
| Glucose | + | 3/4 | 4 | + | 2/2 | 2 | + | 7/8 | 4 | + | 4/5 | 2 |
| Galactose | + | 3/4 | 4 | ++ | 2/2 | 2 | trace | 6/8 | 4 | — | 0/5 | 2 |
| "Mannose" | + | 1/4 | 4 | + | 1/2 | 2 | trace | 1/8‡ | 4 | + | 1/5 | 2 |
| Pentose sugar | + | 3/4 | 4 | + | 2/2 | 2 | + | 1/8‡ | 4 | — | 0/5 | 2 |

* Symbols are used as follows: —, none; +, small amount; ++, moderate amount.
† Frequency indicates No. of times material was present over total No. of relevant chromatograms.
‡ The one sample of CSA that showed a trace of mannose and pentose sugar had been paper electrophoresed and eluted before hydrolysis and chromatography.

rechromatographed, xylose was present in the material from fraction 1 and arabinose in material from fraction 2, confirming the presence of galactosamine and glucosamine in the respective fractions.

The results of chromatographic analysis for sugars and uronic acids from fraction 1 (4 solvent systems) and fraction 2 (2 solvent systems) are summarized in Table I. A uronic acid with same mobility and same color as glucuronic acid after aniline hydrogen phthalate staining and differing from galacturonic acid was present in hydrolyzates from fraction 1. The Dische procedure(11) confirmed the presence of uronic acid in fraction 1. No uronic acid was seen on chromatograms of fraction 2. The results of application of the Dische procedure to fraction 2 were somewhat equivocal; in a sample of fraction 2 several times larger than that of fraction 1, there was a small peak in absorption curve at 530 m$\mu$, the peak for uronic acids. However, at no time during the procedure was the color of this sample like that of any other samples tested, which included fraction 1, CSA, and sugars. If uronic acid was present, the compound of which it was a part must have been only a minor component of fraction 2.

Small amounts of glucose were found in all samples, including the resin blank. (A strong glucose spot was present in samples obtained by subjecting a few fibers of Kleenex to the hydrolytic and chromatographic procedures). About twice as much galactose was seen in fraction 2 as in fraction 1; it was sometimes present in small amounts in CSA but never in the resin blank. A faint spot with the $R_f$ value of mannose or arabinose was seen on chromatograms of fractions 1 and 2 run in the butanol-pyridine-water solvent system but not in the Fischer-Dörfel system. Another sugar with the $R_f$ value of lyxose or xylose and giving the red color reaction to aniline hydrogen phthalate characteristic of pentose sugars was always present in samples of fractions 1 and 2. It was not found among hydrolysis products of CSA, except in one case when CSA was electrophoresed prior to hydrolysis. An eluate of a large amount of paper that had been carried through the electrophoresis procedure, was hydrolyzed, and the products chromatographed. There was a barely perceptible spot at the xylose position. Therefore, the pentose sugar found in the mucopolysaccharide hydrolyzates does not come from the paper but is either a component of the mucopolysaccharides or a breakdown product.

*Discussion.* The major constituents of fraction 1 were glucuronic acid, galactosamine, and sulfate, the components of CSA. In addition, fraction 1 behaved like CSA when electrophoresed and chromatographed. The reason for presence of small amounts of other sugars has not been explained, but they were also sometimes found in commercial samples of CSA. It seems likely that at least the major part of fraction 1 is chondroitin sulfate.

Attempts to identify fraction 2 have been less successful, partly because very small amounts of this fraction have been available for study. In addition to its staining with Alcian blue, the presence of glucosamine in fraction 2 identifies it as a mucopolysaccharide. Its paper-electrophoretic mobility did not coincide with that of CSA, heparin, or hyaluronic acid, although it did migrate just in front of hyaluronic acid in one system and just behind it in another. Galactose appeared prominent, and some other sugars may be present in small amounts. Polysaccharides without uronic acid containing glucosamine, sugars, and no uronic acid have been reported in calf bone(12,13), in calf and steer bone (14), and in connective tissue(15). Their presence in human costal cartilage has also been suggested(16). The blood group polysaccharides have a similar composition. The sugars most often found in these substances are galactose, mannose, and fucose.

The functional significance of the fractions described in this account is not known, but several possibilities have been suggested(1).

*Summary.* Two mucopolysaccharide fractions have been extracted from blood platelets of rats. Paper electrophoresis and paper chromatography of the intact MPS of fraction 1 and paper chromatography of its hydrolysis products show it to be similar to chondroitin sulfate. Fraction 2 is probably a uronic acid-free mucopolysaccharide.

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# Studies on Specificity of Streptokinase.*† (24495)

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The specificity of streptokinase (SK) has been studied by a number of investigators(1-6) and possible explanations for its inability to activate plasminogen of certain animal species have been presented (7a, 7b, 7c, 8, 9). Geiger(10) suggested that SK required a complement-like factor present in human blood to activate guinea pig plasminogen which is not activatable by SK alone. Subsequent studies (11-14) have shown that activation of plasminogen by SK is preceded by reaction of SK with a proactivator, to form activator. It was also shown that the activator not only activated human plasminogen, but was also able to activate bovine plasminogen and the plasminogen of other species of animals(4) refractory to activation by SK alone. The purpose of this paper is to report the following: (a) When the plasminogen of a given species is activatable by SK, the SK-plasminogen mixture gains ability to activate plasminogen of a species refractory to SK. (b) The extent of activation observed in the activatable species seems to parallel ability of the SK-plasminogen mixture to activate a species of plasminogen refractory to SK.

*Materials and methods. Plasmas* were obtained from citrated blood of monkey, horse, sheep, goat and cow and from pooled citrated blood of cat, dog, rabbit, pig, guinea pig and man. *Euglobulin fractions* were prepared from aliquots of each plasma sample as described by Milstone(15) and resuspended at concentration 4 times that of original plasma sample. Guinea pig euglobulin was resuspended at 10 times its plasma concentration. *Human plasmin* was prepared by the method of Sherry and Alkjaersig (7c). *Lysine ethyl esterase activity* was measured as described previously (9) by incubating various dilutions of euglobulin with 30,000 units of SK‡ and 84 μMoles of lysine ethyl ester (LEE) for 60 minutes at 37°C. One unit of lysine ethyl esterase (LEEase) activity hydrolyzes 0.84 μMoles of LEE (1% hydrolysis) under the conditions of the assay. *Activator activity* was measured by adding 35 mg of a bovine plasminogen preparation(9,16) to lysine ethyl esterase test system. Any increase in esterase activity was considered to be due to activation of the bovine plasminogen by the SK-euglobulin mixture, since concentration of SK or euglobulin employed do not individually activate bovine plasminogen. *Plasma fibrinolytic activity* was determined by adding 0.9 cc of plasma diluted with equal volume of borate buffer to 0.1 cc SK, clotting the mixture with 0.1 cc rabbit clotting globulin and recording the lytic time during incubation at 37°C. Reagents used in this test are described by Christensen(17).

*Results.* 1. *SK activation of plasminogen of various species of animals.* (*a*) *Fibrinolytic activity of plasma after SK addition.* Results are tabulated in Table I. In the absence of SK all clots were stable for at least 72 hours.

(*b*) *LEEase activity of SK activated euglobulins.* Extent of activation of plasminogen by SK was also determined by measuring LEEase activity of the formed activator and plasmin(13,14,9,16). To reduce the inhibitor(s) content and to concentrate potentially available enzymes, the euglobulin fraction of each species was used. Results obtained are also shown in Table I.

The data indicate that ability of SK to induce clot lysis when added to a plasma, parallels its ability to induce LEEase activity when added to corresponding euglobulin fraction.

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† The authors are grateful to Miss J. L. Prideau for assistance in certain experiments and to Dr. R. J. Price for supplying plasmas of the various species. We also thank Dr. E. C. De Renzo for helpful discussions and suggestions in preparation of this manuscript.

‡ SK added as Varidase®—Streptokinase-Streptodornase (Am. Cyanamid Co.).

TABLE I. SK Activation of Plasminogen of Various Species of Animals.

| Species of plasminogen | Fibrinolytic activity* | | LEEase activity‡ | |
|---|---|---|---|---|
| | 600 u SK | 6000 u SK | No SK | 30,000 u SK |
| Human | incoag.† | | <1.5 | 130 |
| Cat | " † | | <6.0 | 130 |
| Dog | ca. 1 min. | | 8.5 | 92 |
| Monkey | " | | <2.0 | 78 |
| Rabbit | " | | <6.0 | 35 |
| Horse | no lysis | 2 to 4 hr | <2.0 | 7 |
| Pig | " | ca. 24 hr | <2.0 | 0 |
| Sheep | " | no lysis | 0 | 0 |
| Cow | " | " § | <2.0 | 0 |
| Goat | " | " | 0 | 0 |
| Guinea pig | " | " | 9.0 | 17 |

* Values show clot dissolution times.

† Such large amounts of plasmin formed that defibrinogenation probably occurred.

‡ Values expressed as units of LEEase activity/ml of concentrated euglobulin.

§ Addition of 200,000 units of SK to cow plasma resulted in clot lysis indicating activation of bovine plasminogen. However, activation is very weak since clot lysis results only after 4 to 12 hr.

2. *Activator activity of SK-euglobulin mixtures of various species.* The above findings suggested that those plasminogens activatable by SK might be endowed with a corresponding activator activity in presence of SK. Mixtures of various dilutions of euglobulins and 30,000 units of SK were assayed for LEEase activity in the presence or absence of bovine plasminogen.[§] Those species activatable by SK displayed activator activity (Fig. 1). Those species incapable of activation by SK showed no activator activity (data not shown). Even though guinea pig euglobulin was slightly activatable by SK[||] no activator activity was observed.

3. *Activation of plasminogens not activatable by SK, by a mixture of SK and human plasminogen.* Fig. 1 shows that the SK-human euglobulin mixture appeared to be the most effective activator of bovine plasminogen. It was of interest, therefore, to determine ability of this mixture to activate those species of plasminogens not activated or only poorly activated by SK. The procedure was essentially that used in previous experiment except that the activating mixtures were composed of 30,000 units SK and 7.5 or 15 mg of dried human plasma (1 cc of plasma yielded 125 mg of dried powder on lyophilization). Bovine plasminogen used in experiments shown in Fig. 1 was replaced by 0.5 ml of the euglobulin fraction of the species to be activated. The results are summarized in Table II.

These results are in accord with those reported in the literature(4,5,12,14) and indicate that a mixture of SK and human plasma is capable of activating those species of plasminogen not activatable by SK alone.

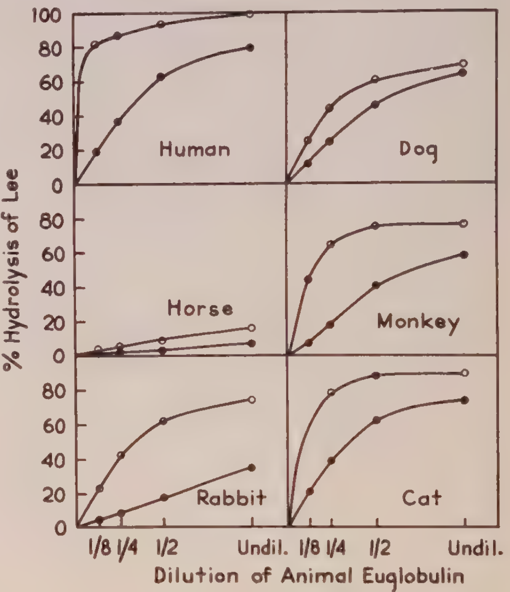

FIG. 1. LEEase (●) and activator (○) activity of a mixture of SK and animal euglobulins (see text for details of assays).

4. *Inhibition of human plasmin by various animal plasmas.* Ability of glycerol activated human plasmin (7c) to hydrolyze LEE in the

TABLE II. Activation of Animal Euglobulins by a Mixture of SK and Human Plasminogen.

| Species of euglobulin | % hydrolysis of LEE* | |
|---|---|---|
| | 15 mg human plasma | 7.5 mg human plasma |
| Horse | 48 | 45 |
| Sheep | 24 | 15.5 |
| Pig | 18 | 14.5 |
| Goat | 12.5 | 3.0 |
| Guinea pig | 74.5 | 43 |

* LEEase activity due to SK and human plasma alone was subtracted to obtain values shown.

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[§] pH optimum and effect of SK and/or euglobulin concentration for each species were not determined.

[||] It should be recalled that bovine and pig plasminogens are also weakly activatable by SK and similarly do not display activator activity.

TABLE III. Inhibition of Human Plasmin by Various Animal Plasmas.

| Species of plasma added to human plasmin and LEE | Units of LEEase activity | % inhibition |
|---|---|---|
| None | 336 | |
| Human | 216 | 36 |
| Dog | 168 | 50 |
| Sheep | 144 | 58 |
| Cow | 96 | 72 |
| Horse | 84 | 75 |

presence of plasma of various species was also investigated. The plasmin assay using LEE has been described(9). An equivalent amount of each plasma was added to various dilutions of plasmin and these mixtures were assayed for LEEase activity. The extent of inhibition of human plasmin by each plasma is tabulated in Table III. The plasma of all species studied showed a partial inhibition of plasmin activity. The quantity of inhibitor in a particular plasma must therefore determine in part the observed extent of activation of a given species of plasminogen.

*Discussion.* The data of our experiments show that the degree of fibrinolytic and LEEase activities induced by addition of SK to a given species of plasminogen parallel each other. That addition of small amounts of human plasminogen to a species of plasminogen refractory to SK activation leads to activation of the refractory plasminogen (Table II) is in accord with observations from many laboratories(4,5,10,11,12,13,14,19). Our experiments also show that any species of plasminogen activatable by SK forms, upon mixture with SK, an activator of a refractory plasminogen (Fig. 1).

The following reactions have been proposed to represent activation of plasminogen by SK: 1. SK + proactivator (X) $\longrightarrow$ activator (SKX) 2. Activator (SKX) + plasminogen $\xrightarrow{\text{enzymatic}}$ plasmin. Astrup and Müllertz and their associates(8,18) and others(13,14) have suggested that inability of SK to activate plasminogen of certain species might be due to a deficiency of proactivator. This theory considers proactivator to be distinct from plasminogen. As a result of studies on the nature of proactivator and plasminogen in human plasma fractions, a simpler hypothesis was proposed in which plasminogen and proactivator were considered to be identical (7a, 9,19). Alternative suggestions, other than proactivator deficiency, were advanced to explain the inability of SK to activate refractory plasminogens.

Unfortunately, the data obtained in the present studies do not help preferentially to support or eliminate either of these concepts. Two other points of possible interest appear worthy of comment. First, the net plasmin activity observed for a given plasma sample must depend in part on the amount of plasmin inhibitor(s) present (Table III). Second, activation (Table I) of refractory plasminogens by large amounts of SK suggests that an inhibitor(s) of SK must be overcome before activation can occur. If the reaction depicted in equation (1) above is valid, small amounts of SK might be expected to activate, albeit weakly, a poorly activatable species of plasminogen. The presence or absence of SK and/or activator inhibitors and not the concentration of proactivator could be a primary factor governing the activation of a given species of plasma or plasma fractions by SK.

*Summary.* 1. Ability of SK to activate the fibrinolytic system in plasma or euglobulin fractions of various animal species has been studied. 2. Human, cat, dog and rabbit plasminogens were readily activated by SK. Pig, horse and cow plasminogens were much less readily activated. Sheep, guinea pig and goat plasminogens were not activatable by concentrations of SK employed. 3. When SK is able to induce formation of a fibrinolytic system on addition to a given species of plasma or euglobulin fraction, that particular mixture gains ability to activate plasminogen of an animal species not activatable by SK alone. 4. Mixtures of SK and a species of plasminogen in which fibrinolysis or LEEase activity does not occur are incapable of activating plasminogens refractory to SK activation. 5. Addition of plasma from all animal species studied partially inhibits the LEEase activity of a spontaneous plasmin preparation. 6. Possible implications of these findings are briefly discussed.

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## Suppression of Acoustic Input by Thalamic Stimulation.* (24496)

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Studies by Hernandez-Peon and others(1,2) on conscious cats with chronically implanted electrodes have shown that voltage of the response evoked in the cochlear nucleus by a standard sound decreases reversibly when the animal's attention shifts from acoustic to other sense modalities. It so appears that irrelevant information can be prevented from entering the brain further than the first relay nucleus on the afferent path. The centrifugal pathways thrown into operation under such circumstances and which must convey the influence of higher levels of the nervous system to the gating process are unknown. The suggestion is currently put forward that the brainstem reticular formation, which is known to regulate the excitatory state of the cerebral cortex(3), might also exert a direct control over sensory input(*e.g.* 4). We feel however that a good case can be made for distinguishing the neurophysiological processes underlying attention and perceptual selectivity from the reticular arousal mechanism, the latter being non-specific in its intrinsic operation. Direct evidence in favor of such a dissociation has been provided by acute experiments on cats showing that cochlear nucleus response to sound could be suppressed by brainstem stimulation(5,6). The anatomical distribution of electrode locations from which such inhibitory effects were observed was quite restricted, and it was certainly not coextensive with the reticular formation. Indeed the positive points were situated laterally to the accepted limits of the reticular formation and in the close vicinity of the lateral lemniscus and inferior colliculus(6). The structures stimulated in these experiments apparently represent links on a very specific acoustic descending inhibitory pathway which, as shown in the present paper, can be traced further cephalad in the diencephalon.

*Methods.* The experiments were made on adult cats immobilized with decamethonium or flaxedil (gallamine) and artificially ventilated. The initial surgical preparation was performed under ether anesthesia. Skin incisions were infiltrated with procaine. Electrical stimulation of deep structures in the brain and recording from the cochlear nu-

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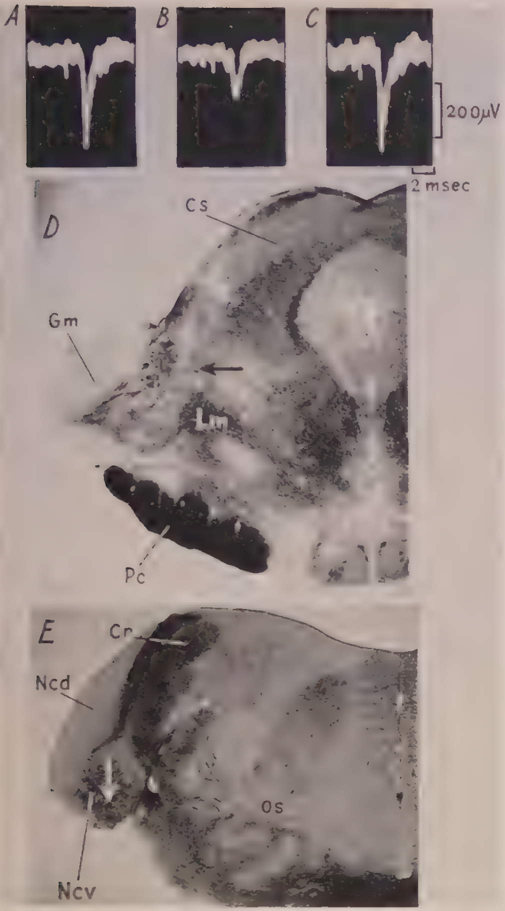

FIG. 1. Cat A 84, 3.2 kg. *A*, *B* and *C*, cathode-ray oscillograms of responses of right ventral cochlear nucleus to a near-threshold click. Four to 5 sweeps have been superimposed in each record to eliminate random fluctuations in response amplitude. Negativity of active recording electrode drives the trace upward. *A*, before, and *B*, during stimulation of inhibitory point in right diencephalon with shocks of 0.5 msec., 6 volts at 150/sec. *C*, just after faradization. *D*, frontal section in an oblique 45° plane (see 7). Spielmeyer method for myelin. Arrow points to trace of stimulating electrode. *E*, frontal section in 45° oblique plane through posterior part of ventral cochlear nucleus to show location of recording electrode (arrow). Spielmeyer method.

*Abbreviations.* Bci, brachium colliculi inferioris. Ci, colliculus inferior. Cr, corpus restiforme. Cs, colliculus superior. Gm, nucleus geniculatus medialis. Lm, lemniscus medialis. Ncd, nucleus cochlearis dorsalis. Ncv, nucleus cochlearis ventralis. Os, oliva superior. Pc, pedunculus cerebri.

cleus were effected with bipolar stainless steel needles oriented stereotaxically. These electrodes tapered to about 50 μ and were insulated to the tip with varnish. The electronic equipment included Grass stimulator model S4C, differential preamplifier and DuMont oscilloscope. Monaural acoustic stimulation was performed with click emitted by a PDR-10 earphone. The sound was directed to the ear-drum through a short rubber tube and the hollow ear-bar of the stereotaxic instrument. Precise anatomical control of electrode positions was facilitated by depositing iron electrolytically from the electrode tip at critical points. After completion of experiment, the cat's head was perfused with 10% formaline, ferrocyanide being added to give a prussian blue reaction with the iron. Serial sections were prepared from each brain and stained with the Nissl and Spielmeyer methods.

*Results.* In our current electroanatomical study of acoustic centrifugal pathways, we try to delimit for each frontal level in the brain, the regions, stimulation of which will suppress acoustic input recorded at the first relay nucleus. The test used is the potential wave evoked in the ventral cochlear nucleus by a near-threshold homolateral click (Fig. 1,A). No attempt is made to isolate cochlear units in the records. Then trains of electrical stimuli at 100-200/sec are delivered to a separate electrode inserted (vertically in the present series) into the diencephalon. This electrode is moved in millimeter steps to explore the region systematically until an inhibitory effect on cochlear nucleus response to click is recorded (Fig. 1,B). As a rule inhibition is not observed after a single shock. It appears during faradization and dissipates quickly when stimulation is withdrawn (Fig. 1,C). Another similar experiment on a different cat is illustrated (Fig. 2, A to C) to show that clear-cut suppression of acoustic input can be consistently produced by repetitive stimulation of a critically-localized area in the posterior diencephalon. This area (Figs. 1,D and 2,D) does not correspond to a readily definable anatomical structure and it certainly includes fiber tracts and cell components which remain to be elucidated. It is situated on the mesial aspect of the nucleus geniculatus medialis and dorso-laterally to the lemniscus medialis and to the rostral reticular formation. The region is very restricted and does not belong to the classical auditory pathway in the sense that clicks generally evoke no potential from it.

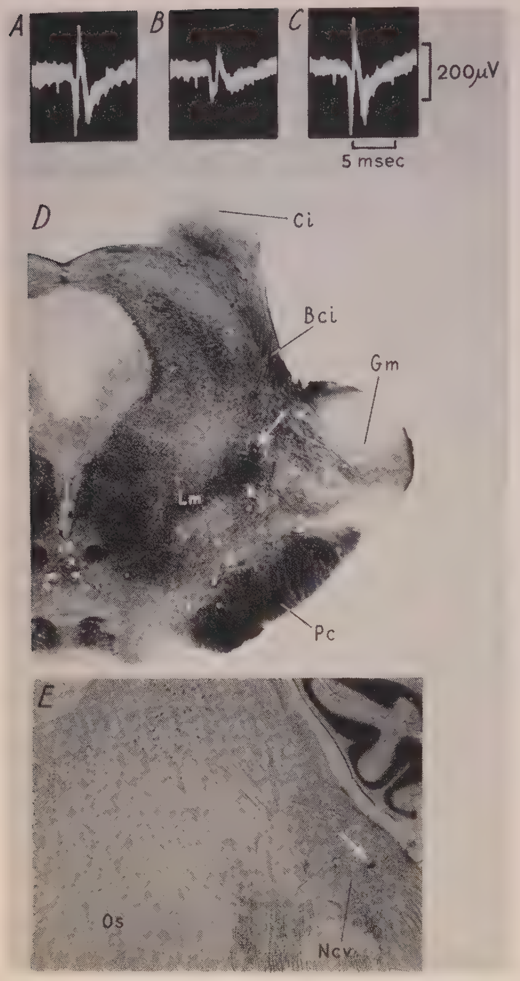

FIG. 2. Cat A 99. 2.8 kg. *A*, *B* and *C*, oscillograms of 4-5 superimposed responses of left ventral cochlear nucleus to a near-threshold click. *A*, before, and *B*, during faradization of inhibitory point in left diencephalon with shocks of 0.5 msec, 7 volts at 150/sec. *C*, just after faradization. *D*, frontal section in 45° plane showing trace of stimulating electrode (arrow). Spielmeyer method. *E*, frontal section in 45° plane through middle part of ventral cochlear nucleus showing trace of recording electrode (arrow). Toluidine blue stain.

By contrast, when the thalamic electrode is inserted more laterally in the same frontal plane, acoustic responses can be recorded (in the nucleus geniculatus medialis) but faradization of these points has not suppressed cochlear response.

That the phenomenon recorded in these experiments is genuine inhibition and not occlusion by possible antidromic stimulation of cochlear axons is indicated by the last-mentioned results showing that points stimulated are close to, but not within the ascending auditory pathway. Moreover a single shock applied to these points evokes no antidromic potential in cochlear nuclei, which in fact do not seem to project fibers further cephalad than the midbrain.

As found previously(6) repetitive stimulation of an inhibitory point situated on any one side of the brain suppresses the acoustic potential recorded both in right and left cochlear nuclei. The centrifugal pathway thus possesses commissural connections which enable it to control simultaneously acoustic input from both ears.

*Comment.* Little is known about how sensory messages are translated into conscious perception, but a promising start has been made in this direction by analyzing how the brain deals with messages according to its interest in them. Clearly irrelevant stimuli can be screened out at quite early stages in the afferent pathway(1,2) and the sensory information is only handed over to the cerebral cortex after having been subjected to a sort of "editing which emphasizes the important items and sets the unimportant aside" (8).

The present experiments relate to the problem because they provide the first physiological evidence for a specific descending inhibitory pathway, the function of which is presumably to modulate acoustic input to the brain. The descending pathway has been traced at the level of midbrain(6), of the posterior diencephalon (this paper) and further cephalad (to be published). It seems very restricted and the stereotaxically oriented electrodes have to be placed at critical points to produce the effect. In some experiments no inhibition was seen although the preparation seemed in good condition; in many of these cases anatomical study of the brain showed subsequently that the stimulating electrode had narrowly missed the critical region and had been inserted somewhat more medially or laterally.

The evidence indicates consistently that we are dealing with a quite specific descending pathway which seems to be operated by higher levels of the brain. It does not substantiate the hypothesis that the brainstem reticular formation might control acoustic input through direct reticulo-cochlear fibers.

*Summary.* 1. Electrical responses to clicks were recorded from the ventral cochlear nucleus in curarized cats, and the effect thereon of brain stem repetitive stimulation was investigated (stereotaxic method). 2. Clear-cut inhibition of the cochlear nucleus response to click was recorded when a critically-localized region of the posterior diencephalon was stimulated. 3. These and previous results provide the first physiological evidence for a specific extra-reticular descending pathway, presumably enabling higher levels of the nervous system to control acoustic input.

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## Paper Electrophoresis of Duodenal Fluid from Patients with Cystic Fibrosis of Pancreas. (24497)

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Farber(1) suggested that the basic pathology in patients with cystic fibrosis of the pancreas resulted from abnormal mucous secretion. In patients with meconium ileus, a common manifestation of cystic fibrosis, the meconium has been demonstrated chemically by Rapoport and Buchanan(2) to contain an abnormal mucoprotein and electrophoretically by Green, Clarke, and Shwachman(3) to have an abnormal protein component. Recently di Sant' Agnese *et al.*(4), using precipitation with a benzene-alcohol mixture, have demonstrated an abnormal mucoprotein in the duodenal fluid in children with cystic fibrosis.

In our studies, samples of duodenal fluid from children with cystic fibrosis were examined by paper strip electrophoresis and the results compared with those observed in control children.

*Methods.* Ten children with cystic fibrosis were studied, 6 with this diagnosis considered but ruled out by laboratory studies, and 2 normal children. Following intubation, in most instances with fluoroscopic or x-ray verification of the position of the duodenal tube, duodenal fluid was collected in an ice bath. After centrifugation the fluid was frozen until used for electrophoretic study. A volume of .04 cc of the fluid was applied to Schleicher and Schuell (No. 2043A-mgl) filter paper strips in Spinco Model R electrophoresis cell. A veronal buffer of pH 8.6 and ionic strength 0.075 was used with constant current of 2½ ma applied for 16 hours. Following electrophoresis the strips were dried at 120°C for 30 minutes, fixed in absolute methanol for 6 minutes, stained with Bromphenol Blue Dye (Spinco) in alcoholic solution for 30 minutes, rinsed 3 times in 5% acetic acid, dried at 120°C for 15 minutes, and placed in ammonia fumes for 15 minutes-4 hours to enhance color development. Then they were read immediately with Spinco RA Analytrol, utilizing 1½ mm slit, 500 mu interference filters, and a B-5 cam.

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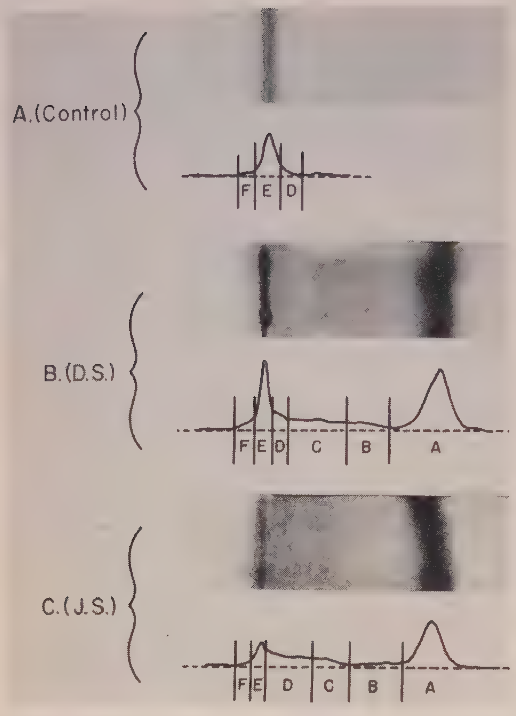

FIG. 1. Duodenal fluid protein pattern: (A) Normal, (B) and (C) cystic fibrosis.

*Results.* The duodenal fluid from each patient with cystic fibrosis had an abnormal electrophoretic component not present in any samples obtained from the 8 control subjects. Fig. 1 shows typical electrophoretic patterns obtained on duodenal fluid from a normal child and from 2 children with cystic fibrosis. In each instance a photograph of the paper strip and a reproduction of the analytrol pattern is shown. The peak labeled E represents the point of application of the sample to paper strip and the other peaks are designated arbitrarily as F, D, C, B, and A. In duodenal fluid samples from the 8 control children there was but little electrophoretic migration and only the peaks F, E and D were found. On the other hand, in each sample obtained from 10 patients with cystic fibrosis there was migration past the D fraction, the major portion of this more rapidly migrating material appearing in fraction A. The electrophoretic mobility of this material in fraction A was identical with that of the albumin fraction in normal human serum by comparing patterns obtained when samples of normal serum and cystic fibrosis duodenal fluid were submitted to electrophoresis simultaneously under identical experimental conditions.

The test of di Sant' Agnese *et al.*(4) for abnormal mucoprotein in duodenal fluid was performed on most of the cystic fibrosis patients and consistently was found to be positive. However, there was no correlation between quantity of abnormal mucoprotein and quantity of abnormal protein component A in individual duodenal fluid samples. In addition, periodic-acid-Schiff staining of strips revealed no difference between normal subjects and cystic fibrosis patients with regard to distribution of mucoproteins in the duodenal fluid.

*Discussion.* Although there is little information available regarding electrophoresis of human duodenal fluid, several electrophoretic studies of pancreatic juice in rats and dogs have been reported(5-9). In contrast to the small number of protein fractions in the duodenal fluid from human controls in our investigation, the studies in animals all demonstrated 4-7 fractions. Munro and Thomas (5) found that none of the fractions obtained by free electrophoresis of canine pancreatic fluid correlated with the albumin fraction in serum but that the fractions did appear to correlate with the globulin serum fractions, alpha-1, alpha-2, beta, and gamma respectively.

The fast moving fraction A in cystic fibrosis duodenal fluid may correspond to the abnormal peak found by Green *et al.*(3) in meconium of patients with meconium ileus; this latter peak also had a mobility identical with the albumin peak of normal human serum. In their study and ours evidence was obtained indicating that the rapidly migrating abnormal protein fraction demonstrated by electrophoresis does not correspond to the abnormal mucoprotein described by di Sant' Agnese *et al.*(4) in duodenal fluid of patients with cystic fibrosis. Although the identity of the material in fraction A with serum albumin has not been proved, the evidence strongly suggests that the proteins in duodenal fluid of cystic fibrosis patients are serum proteins. The possibility should be considered that all duodenal fluid originally contains serum proteins but in the normal individual these are hydrolyzed rapidly by pancreatic enzymes, whereas in cystic fibrosis patients such hydrolysis does not occur and the serum proteins remain detectable in the duodenal fluid.

Regardless of the exact nature of the material represented by the faster moving electrophoretic components of duodenal fluid from cystic fibrosis patients, the mere existence of these components is of clinical significance. If further study confirms the observation reported here that a difference regularly exists between the electrophoretic patterns of duodenal fluid in cystic fibrosis patients and those in normal subjects, an additional simple laboratory test will be available for use in establishing the clinical diagnosis of cystic fibrosis.

*Summary.* Duodenal fluids from patients with cystic fibrosis of the pancreas and from control subjects were studied by paper strip electrophoresis. Components with greater electrophoretic mobility than any in normal duodenal fluid occurred consistently in cystic fibrosis duodenal fluids. The most rapidly migrating of these fractions was identical in electrophoretic mobility to the albumin fraction of normal human serum.

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# Adaptation of Calcein Titration Method for Calcium Determination in Tissues Without Ashing.* (24498)

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Determination of calcium in a large number of serum and tissue samples would be facilitated by a method that is simple and rapid, and does not require the cumbersome ashing of tissues in a platinum crucible. Recently several titration methods(1,2,3) have been published but they still require ashing of tissues. The present study was undertaken to adapt the calcein (a fluorescent dye) titration method of Diehl and Ellingboe(4) as modified by Ashby and Roberts(1) to determination of calcium in tissues and milky lipemic serum without the necessity of ashing. Since minor modifications were made and additional steps added, the entire procedure is described for the sake of continuity.

*Materials.* (1) Titration box. Titration end-point of light green fluorescence is seen in the dark under ultraviolet light as the only illumination. Since a dark-room is not always available, a cardboard box, 2 x 3½ x 5 inches in dimensions, has been modified for this purpose and is shown in Fig. 1. (2) A 1 ml microburette, reading to the fourth decimal place; (3) a long-wave ultraviolet lamp; and (4) a piece of PE 10 polyethylene tubing attached to 27 gauge needle which in turn is attached to compressed air line and is used for mixing during titration. All glassware is preferably rinsed in dilute acid followed by a thorough rinsing with distilled water to avoid calcium contamination.

*Reagents.* (1) Concentrated $HNO_3$, reagent grade; (2) 1 N $HNO_3$; (3) 1.5 N KOH; (4) saturated ammonium oxalate solu-

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* The author is indebted to Mr. F. A. Smith for serum calcium determinations by the Clark-Collip method.

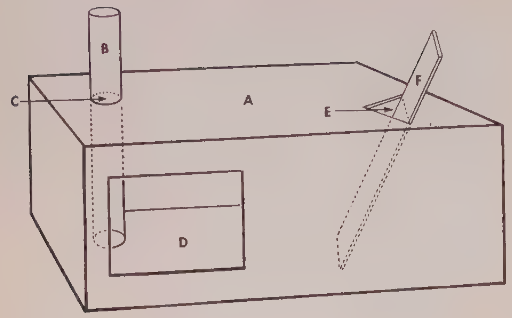

FIG. 1. A, cardboard box 2 × 3½ × 5 inches, painted black inside; B, titration tube; C, ½ inch diameter hole 2 inches from front of box, into which titration tube fits snugly; D, opening in front of box to admit ultraviolet light; ultraviolet lamp should completely cover opening so as to prevent entrance of outside light; E, opening in front of mirror; F, mirror to reflect titration.

tion; (5) concentrated $NH_4OH$; (6) methyl orange pH indicator solution; (7) approximately 0.001 M disodium dihydrogen ethylenediamine tetraacetate (EDTA) solution; (8) calcein indicator solution: dissolve 8 mgm calcein in 0.2 ml of 1 N NaOH and then add water to make total volume of 100 ml; (9) standard calcium solution for titration: 100 $\mu$g Ca/ml; (10) 2 N NaOH solution containing 1% NaCN; (11) n-octyl alcohol. All reagents are prepared with redistilled Ca-free water.

*Procedure.* (1) *Tissue preparation.* Into a small test tube, graduated at 1 ml, place 0.5 ml of 1.5 N KOH and a known weight of tissue or volume of serum and heat in water bath at about 80°C until test material is digested. For normal rat tissues we used about 250 mg liver, 100 mg aorta and 0.1 ml serum/ml of KOH digest to obtain adequate calcium concentration for analysis. After the tissue is digested, add 0.1 ml of concentrated $HNO_3$ and sufficient redistilled Ca-free water to make a total volume of 1 ml, mix with a footed glass rod and let stand for about half an hour. Mix again and centrifuge. Transfer a known aliquot of supernate, 0.8 ml or less as required, to a clean test tube, 14 x 100 mm, add 1 drop of methyl orange indicator and make alkaline to the indicator by dropwise addition of concentrated $NH_4OH$. Add 1 ml of saturated ammonium oxalate, mix and let stand overnight. The next morning centrifuge and remove the supernate by aspiration, being extremely careful not to disturb the precipitate. Do not use an angle centrifuge because this makes it difficult to remove the supernate without losing some of the precipitate. Add 0.5 ml of 1.5% $NH_4OH$ to wash the precipitate and again centrifuge and remove supernate as before. Add 2 drops of 1 N $HNO_3$ and heat gently to dissolve calcium oxalate precipitate. (2) *Titration.* Place tube into titration box and illuminate with ultraviolet light. Add 1 volume of EDTA solution and 1 ml of calcein indicator solution. It is absolutely essential that the identical volume of EDTA solution be delivered each time. This is readily accomplished with a ½ ml hypodermic syringe fitted with 24 gauge needle. Add 1 drop of n-octyl alcohol to prevent foaming and mix by bubbling air through the solution by the polyethylene tubing. Add 5 drops of 2 N NaOH solution containing 1% NaCN. If necessary, repeat EDTA addition until fluorescence disappears, indicating that all the calcium has formed a complex with EDTA. Add sufficient redistilled Ca-free water to make final volume of EDTA solution plus water 2 ml. Now titrate with standard calcium solution until a light green fluorescence persists, indicating an excess of free calcium in solution. Also titrate directly 1 volume of EDTA solution to standardize the EDTA solution and use a reagent blank carried through the entire procedure to check reagent contamination with calcium. The

TABLE I. Serum Calcium Determinations.

| Calcium conc., mg/100 ml | | | |
|---|---|---|---|
| Normal | | After parathyroid hormone | |
| Clark-Collip method | Present procedure | Clark-Collip method | Present procedure |
| 10.40 | 10.42 | 14.60 | 14.60 |
| 10.60 | 10.98 | 13.40 | 13.68 |
| 10.90 | 10.57 | 15.40 | 15.06 |
| 11.30 | 11.36 | 15.50 | 15.98 |
| 10.90 | 11.38 | 14.00 | 14.36 |
| 10.90 | 10.86 | 13.80 | 14.02 |
| 10.90 | 11.39 | 15.00 | 15.48 |
| 11.00 | 11.52 | 15.00 | 14.50 |
| 10.00 | 10.17 | 12.70 | 14.01 |
| 11.20 | 11.76 | 14.50 | 16.01 |
| 10.81* | 11.04 | 14.39 | 14.77 |
| ± .20 | ± .28 | ± .28 | ± .25 |

* Mean ± S.E.

amount of calcium titrated is then calculated by difference.

*Results.* For preparation of the tissue it was essential to obtain a relatively clear supernate and to insure complete solution of calcium present. To satisfy the latter requirement an acid solution was desirable. Trichloroacetic acid, sulfuric acid, and hydrochloric acid interfered with titration, but nitric acid gave satisfactory results.

For convenience, the validity of the present procedure was investigated in 2 steps. First, determination of calcium in dog serum was compared with the Clark-Collip method(5) as outlined in the USP XV(6) for assay of parathyroid hormone. The results (Table I) show good agreement. Secondly a tissue KOH digest was prepared. An aliquot of this was ashed in a platinum crucible in the usual manner. After ashing, the residue was dissolved in $HNO_3$, then analysed for calcium by the present procedure, starting with the oxalate precipitation. A second aliquot was processed as outlined above. The results presented in Table II show that tissue ashing was not necessary.

Further studies indicate that good recovery of calcium, added to the tissue just prior to KOH digestion, was obtained. These results are presented in Table III.

TABLE II. Tissue Calcium Determination.

| Liver calcium, mg/100 g wet tissue | |
|---|---|
| With ashing | Without ashing |
| 5.80 | 5.55 |
| 2.93 | 3.02 |
| 4.06 | 4.36 |
| 4.71 | 4.15 |
| 5.85 | 5.23 |
| 6.82 | 5.04 |
| 3.76 | 4.59 |
| 6.10 | 5.15 |
| 3.78 | 3.20 |
| 4.18 | 4.31 |
| 5.32 | 5.43 |
| 5.55 | 5.65 |
| 4.90 ± .35* | 4.64 ± .25 |

* Mean ± S.E.

TABLE III. Recovery Studies.

| | | With ashing | | Without ashing | |
|---|---|---|---|---|---|
| Tissue | µg calcium added | µg recovered | % recovery | µg recovered | % recovery |
| Liver | 10 | 9.0 | 90 | 8.8 | 88 |
| | | 10.2 | 102 | 9.7 | 97 |
| | | | | 9.0 | 90 |
| | | | | 9.1 | 91 |
| | 20 | 19.6 | 99 | 19.8 | 99 |
| | | | | 20.3 | 101 |
| | 40 | 39.5 | 99 | 39.1 | 98 |
| | | 38.9 | 97 | 35.5 | 89 |
| | | | | 37.9 | 95 |
| | | | | 39.3 | 98 |
| | | | | 37.1 | 93 |
| Aorta | 5 | | | 4.6 | 92 |
| | | | | 4.3 | 86 |
| | 10 | | | 10.5 | 105 |
| | 20 | | | 18.8 | 94 |
| | 40 | | | 38.0 | 95 |
| | | | | 33.2 | 83 |
| Serum | 5 | | | 5.5 | 110 |
| | 40 | | | 38.0 | 95 |
| | | | 97.4 *<br>±2.02 | | 94.7<br>±1.5 |

* Mean ± S.E.

*Summary.* A convenient and rapid procedure is presented for determination of calcium in tissues and lipemic serum without the necessity of ashing.

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# Decreasing "Amino Acid Hunger" of Human Muscle with Age.[*] (24499)

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Recently we reported that the extent to which various tissues of the rat concentrate the non-metabolizable amino acid α-aminoisobutyric acid (AIB) steadily decreases with age (1,2). For example, at 50 g body weight the level estimated for muscle cell water averages over 4.5 times the serum level, whereas at 350 g the ratio is only about 2.5 to 1. AIB was selected for these experiments because it does not enter catabolism or protein synthesis, thereby permitting isolation for study of the transport process, which it enters in typical manner(3,4). The present communication reports a very similar observation in man.

*Methods.* Male patients without apparent disturbances of general metabolism, hydration or circulation were selected, who were also candidates for surgical procedures in which muscle samples probably could be obtained with minimal harm. A sterile solution of 1.43 microcuries (143 μg)/kg body-weight of $C_1^{14}$-labeled α-aminoisobutyric acid in 0.5 to 1.2 ml 0.9% NaCl was injected into the deltoid muscle the day before surgery. This dose represents 100 microcuries of $C^{14}$ (10 mg AIB) for a 70-kilo man, in a form with roughly a one-day biologic half-life and no detectible incorporation into body structures. Whenever surgery permitted, usually from 0.5 to 1.0 g of muscle was removed at 19 ± 1 hrs after injection. (To minimize the radioactive dose necessary and for technical reasons we allowed 19 hrs for the amino acid to be distributed, rather than the 39 hrs allowed in the rat.) A blood sample was taken from an antecubital vein 2 or 3 minutes later. The muscle sample was divided at once into 3 samples, placed in tared weighing bottles. One sample was used for water determination by drying at 110°. The second sample was extracted with 0.75 *M* nitric acid and the chloride determined; by comparison with serum chloride, the content of extracellular and cellular water could be estimated(5). The third portion of muscle was homogenized by the Potter-Elvehjem method, water and 0.1 *N* acetic acid being added to total 10 volumes/g of muscle. The mixture was held at 100° ten minutes, then cooled and filtered to yield an extract of pH about 5. 0.2 ml portions of this extract, or of blood serum, were plated in triplicate on stainless steel planchets and dried, and the disintegrations counted in a Geiger-Müller thin-window counter. Corrections for self-absorption were determined for the tissues used. In a few of the later experiments where muscle was not analyzed, the plasma proteins were precipitated by heating plasma diluted 1:4 at pH 5 and removed before counting. In this case 0.8 ml of extract were plated by applying first 0.4 ml, then taking to dryness before applying a second 0.4 ml portion. The counts per min were referred in each case to one gram of cellular or plasma water.

*Results.* Table I shows that for 3 men in their early fifties, the level of AIB calculated for muscle cell water was about *2* to *4 times* the level for plasma water. In contrast, muscle cells of 2 boys, 2.2 and 3.5 years old, appeared to concentrate the amino acid 11 *times* with respect to the extracellular phase; for a

TABLE I. Distribution of AIB between Serum and Muscle-Cell Water 19 Hours after Injection.

| Age in yr | Serum<br>cpm/g $H_2O$ | Muscle<br>cpm/g $H_2O$ | Ratio |
|---|---|---|---|
| 2.2 | 82 | 903 | 11.0 |
| 3.5 | 121 | 1385 | 11.6 |
| 9.0* | 102 | 630 | 6.2 |
| 6 children, 2–9 | 117 (82–144) | | |
| 50 | 395 | 1500 | 3.8 |
| 54 | 244 | 961 | 3.9 |
| 54 | 636 | 1480 | 2.3 |
| 6 men, 34–54 | 331 (244–636) | | |

* Girl, elective leg amputation.

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[*] Supported in part by grant from Nat. Cancer Inst., N.I.H., U.S.P.H.S.

[†] Successively medical student research fellows in Dept. of Biological Chemistry.

9-year-old girl AIB was concentrated 6.2 times.

The subjects actually showed no great difference in their *muscle* levels of AIB, a finding which is not strange because they all received the same dose on a weight basis and muscle probably is the largest single site of retained amino acid. Instead the age difference is expressed almost entirely in the plasma level necessary to "back-up" the given tissue level. Extensive experimentation with various cells and tissues has established the concept that an ability to retain a given tissue level in the face of a lower extracellular level represents stronger concentrative activity(6-8).

A combination of 2 factors, an infrequency of patients who met the above criteria on the one hand, and reticence on the other, led us to take advantage of the observed striking difference in plasma levels and therefore to omit surgical removal of muscle tissue in the remaining subjects (Table I). These were normal persons or convalescent surgical patients. A mean 19-hr AIB level for plasma water of 117 counts/min/g for the 6 children contrasts with a mean of 331 counts/min/g for 6 men.

Two men excreted during the first 3 hrs 18.2 and 15.6% of the dose, during the second 3 hrs, 6.7 and 8.4%. Total excretion in the first 24 hrs of the first subject was 45.6%, for the second subject 41.7% in the first 19 hrs. A 4-year-old boy L.S. excreted 37.0% of the dose in 19 hrs.

The following technic is proposed for examining partition of AIB in various endocrinologic disturbances: After intramuscular injection all urine is collected for the standard interval of, say, 19 hrs. At this point a plasma sample is taken and counted. The *apparent volume of distribution* is then calculated, *i.e.*, the volume required to contain the AIB not yet excreted if concentration were uniformly that found in plasma. This procedure gave a volume of distribution at 19 hrs 2.6 times the estimated body water for the 34-year-old subject M.S., and 5.7 times the estimated body water for the 4-year-old L.S.

Fig. 1 shows the apparent volume of distribution of residual AIB, compared to calculated total body water content for an 80-kg man who received 190 microcuries of AIB intramuscularly. Average concentration in body water is about 4.5 times the plasma level at 24 hours. The further increase of the apparent volume in the second day may represent gradual entrance into less accessible compartments; it arises at least partly, however, from our neglect of minor routes of AIB loss from the body and therefore an increasing overestimate of the AIB left in the body. Comparison of plasma and urine levels during the second day yields a renal plasma clearance of AIB of 56 ml per minute, indicating that transfer out of the renal tubule back into the plasma, whether mediated or passive, is slower than for normal amino acids and insufficient to establish a gradient in favor of the plasma.

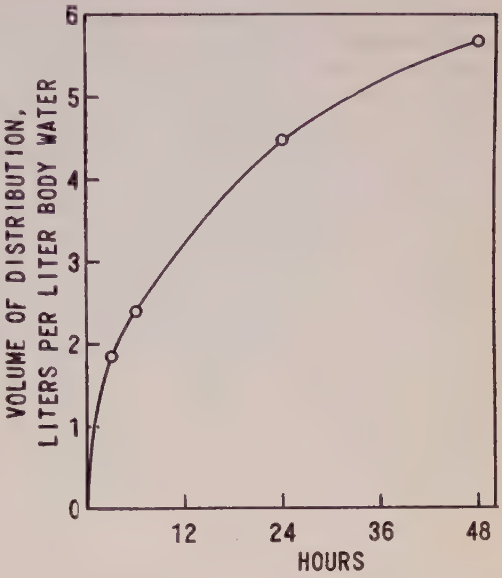

FIG. 1. Gradual increase of apparent volume of distribution of residual AIB remaining in 80 kg man (H.N.C.). Body water was taken to be 68% of body wt. Data on excretion rate appear in text.

The respiratory carbon dioxide was collected as lithium carbonate (4 moles) for the first 6 hours of experiment. A slurry in methanol of the finely ground lithium carbonate was plated to infinite thickness on a planchet and counted for nearly an hour. The count was indistinguishable from background. If we take the maximum count to be one/minute above background, and the plate to represent effectively 25 mg of precipitate/cm$^2$ with a self-absorption of 86%(9), less than 2200 counts/min (or 0.002%) were collected from the 111 million counts/minute injected. This result compares with a less sensitive earlier estimate of maximum catabolism of AIB in the rat of 0.02%(1).

*Discussion.* Although the test amino acid actually was present at very low concentrations, we believe that we should take the effective extracellular level entering the concentration process for most cells to be roughly 2 millimolar, representing the sum of the various neutral amino acids of plasma, since the concentrative process distinguishes only weakly among the neutral amino acids.

The foregoing effect of age was shown for a number of tissues in the rat, and undoubtedly is not limited to muscle in man. Upon stunting rats by calorie-restriction extent of tissue concentration associated itself more closely with body weight than with age(2). According to our interpretation, tissue "amino-acid hunger" is quantitatively associated with potentiality for growth or protein synthesis.

*Summary.* 1. Injected $C_1^{14}\alpha$-aminoisobutyric acid was much more strongly concentrated by skeletal muscle in children than in men. The greater general uptake by tissues could be recognized by the resultant consistently lower plasma level in the young. 2. The absence of catabolism of this amino acid was shown by observation of less than 0.002% of the injected $C^{14}$ in expired air during 6 hours, counting respiratory $CO_2$ in the form of $Li_2CO_3$. After 24 hours average concentration in the body water was about 4 times that in the plasma water. A renal clearance of 56 ml of plasma per min was observed.

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## Changes in Protein and Nucleic Acid Content on HeLa Cells Infected with Equine Abortion Virus.*† (24500)

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Study of the effects of equine abortion virus (EAV) on hamster liver has been reported (1,2). In this host, a rapidly lethal hepatitis usually results in death of the animal within 18 hours and is characterized by very numerous conspicuous intranuclear inclusions. Sequential studies of the infection have shown increased RNA and protein in infected nuclei isolated in citric acid, DNA remaining constant. A search for a better defined, easily attainable and readily susceptible system with which to investigate further the nature of chemical changes induced in hamster liver cells infected with EAV led to the use of HeLa cultures and this adaptation has been reported recently(3). Investigators have utilized tissue culture to demonstrate certain changes in host cell due to virus infection. Kovacs(4) demonstrated marked changes in activities of 9 enzyme systems of poliomyelitis-infected Rhesus monkey kidney epithelium. Maassab,

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‡ Taken, in part, from M.S. thesis of D.J.M.

Loh, and Ackermann(5) reported a sustained high uptake of $P^{32}$ by the nuclear fraction of HeLa cells infected with poliovirus, in which the quantities of nuclear RNA and DNA remained fixed. However, the net synthesis of cytoplasmic RNA was 2.5 x normal. Levy, Rowe, Snellbaker, and Hartley(6) noted a rapid and prolonged stimulation of cellular uptake of radioactivity of inorganic $P^{32}$ and glycine-2-$C^{14}$, with no change in concentration of P, RNA, or DNA of adenovirus type 2-infected HeLa cells. In contrast Boyer, Leuchtenberger, and Ginsberg(7), by means of Feulgen microspectrophotometry, demonstrated an increase in DNA content of HeLa cells infected with adenovirus, types 1 to 4. The data of Newton and Stoker(8) show that HeLa cells infected with herpes after 3-4 days contain double the DNA content of normal cells and little or no RNA, yet number of cells obtained from infected cultures increased rather than diminished. The results reported here are part of a general study of the effect of equine abortion virus on susceptible cells. Certain changes in protein and nucleic acid content of the infected HeLa cell are here reported.

*Methods.* The virus used has been adapted to HeLa cells as noted above(3). *Tissue culture.* The strain of HeLa cells used has been adapted to horse serum in this laboratory for approximately 4 years. Stock cultures were grown in a medium consisting of 20% horse serum and 80% equal parts of mixture #199 of Morgan, Morton and Parker(9) and Earle's balanced salt solution (BSS). Subcultures were established in 250 ml serum bottles and fed every other day. Replicate cultures, 7 days of age, containing approximately 1 x $10^7$ cells, were washed 3 times with BSS to eliminate horse serum. Two or more cultures were inoculated with 1 ml of virus suspension containing approximately $10^3$ to $10^4$ 50% infectious doses ($ID_{50}$) and remained at room temperature for 30 minutes. Controls and infected cultures were incubated at 37° C with 10 ml of a 50-50 mixture of human ascitic fluid and BSS. After 48 hours the infected cultures apparently had undergone complete cytopathic change. Infected cells and maintenance mixture from one bottle of each passage were harvested, frozen and ground. The resulting viral suspension was stored at −50°C in sealed vials. Fresh cultures were inoculated from material of the previous passage. *Enumeration of nuclei.* Aliquots (1/10) of each sample were removed for nuclei counts utilizing the citric acid method of Sanford *et al.*(10). At least 2,000 nuclei were counted in every instance. *Chemical determination.* The remainder of sample containing a minimum of $10^7$ cells was washed 4 times with physiological saline frozen and lyophilized. DNA, RNA and protein were determined in Beckman Model B spectrophotometer using matched silica cells. Methodology for the various chemical determinations has been cited(2). The various procedures were performed on extracts of whole cells rather than on nuclei isolated in citric acid, because of the possible loss of protein and RNA during citric acid isolation as reported by Kay *et al.*(11). Nucleic acids were extracted by the method of Schneider(12) with minor modifications as reported by Bracken and Randall(2). The important advantage of the Schneider procedure as discussed by Webb and Levy(13) is that no separation of DNA from RNA is required since determinations are based on characteristic color reaction for pentoses and desoxypentoses. Relative merits of a number of methods are cited in this review. Diphenylamine reaction of Dische(15) was used to estimate DNA content/cell. The results were compared with similarly tested controls prepared from purified DNA (Mann Laboratories). RNA was determined by the orcinol method of Mejbaum(14). The results were compared with similarly tested standards of commercial yeast nucleic acid (Mann Laboratories). All data were corrected for DNA interference according to Schneider(12) utilizing similarly tested DNA standards. Protein values were determined by a modification of the method of Robinson and Hogden(2). The protein content of whole cell preparations was compared with Armour's protein standard and crystallized edestin (Worthington Biochemical Lab.).

*Results.* The data are summarized and compared statistically in the accompanying

TABLE I. Comparison of Normal and Infected HeLa Cells.*

| | Normal | Infected | △ | P† |
|---|---|---|---|---|
| DNA | 31.0 | 40.2 | + 9.2 | $<.01$ |
| RNA | 40.3 | 41.0 | + .7 | $>.05$ |
| Protein | 336.0 | 525.0 | +189.0 | $<.01$ |

* All values are expressed as mg × $10^{-9}$/cell.

† Probability of obtaining a larger value of t by chance. Level of significance is $P \leqq 0.05$.

Table. Values represent quantities/whole cell. It is interesting to note that whereas DNA and protein are increased significantly, total RNA is not changed during infection. The DNA and RNA control values for uninfected whole cells are similar to that for another type of tissue culture cell, the L cell, as reported by Kuchler, who employed similar methods(16). RNA content of normal HeLa cells is in agreement with the data of Newton and Stoker(8) but their DNA value is about ½ that reported here. The problem of evaluating data arrived at by different methods remains a pressing one.

It was of interest to determine whether any of the material pertinent to the investigation could be determined in the maintenance medium or wash fluid of normal and infected cells. Neither RNA or DNA could be detected in the maintenance fluid by the method employed nor protein in the final saline wash of the cells.

*Discussion.* From our results it is apparent that HeLa cells infected with EAV contain increased amounts of DNA and protein. The latter observation, to our knowledge, has not been reported previously.

Analyses on whole cells have the advantage that the constituents under study can be measured without loss or change that may occur in cell fractionation technics. However, by use of the latter method it would be interesting to compare nuclear and cytoplasmic RNA and protein. The mechanism of the protein increase is unexplained at present. The possibility obtains that during the process of infection altered cell permeability may permit increased amounts of protein from the nutrient fluid to enter the cell, or the change may be attributable to increased protein synthesis.

Relative to the nature of chemical changes induced by viruses, it does not seem wise to equate different viruses or different host cells to one another. However, it is of interest to note that biochemical changes induced by EAV differ in HeLa cells and hamsters. From data on hamster liver cell nuclei DNA apparently remains constant during all stages of infection whereas protein and RNA increase. Admittedly, these data rest on cell fractionation studies and it is possible that there is no overall increase of protein and RNA; at least there is an increase in nuclear content of these constituents. The nature of the increase in DNA relative to the infected HeLa cell, which is malignant, is not known.

As far as HeLa cell is concerned, a number of virus infections are associated with inclusions (adenovirus, EAV, herpes) and apparently result in the induction of increased amounts of DNA. On the other hand, Maassab, Loh and Ackermann(5) have demonstrated increase in cytoplasmic RNA in poliomyelitis-infected HeLa cells.

The question invariably arises as to the composition of the virus in question. The data do not permit an analysis of this problem. It is not likely that intracellular virus particles in themselves would be present in such number as to be detectable by the relatively gross chemical methods employed. The concurring remarks of Newton and Stoker(8) are of interest. Bracken(17) has reported recently an electron microscopy study of EAV in the hamster liver. The small aggregates of intranuclear virus particles are not equivalent to the large intranuclear inclusion visualized by the light microscope.

We have reported increase in certain cellular constituents. It is plausible to suggest that the increased materials are due to altered cell metabolism as a manifestation of virus infection. Elucidation of these phenomena await further investigation of the infected cell and its products.

*Summary.* HeLa cell cultures aged 7 days were infected with equine abortion virus, incubated for 48 hours and compared with control cultures as to content of RNA, DNA and protein. DNA and protein were elevated significantly, whereas RNA was unchanged. The observation that cell protein is increased as a

result of virus infection of a tissue culture system has not been reported previously.

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